QUALITY POTENTIAL OF GLUTEN PROTEINS IN HEXAPLOID WHEAT AND RELATED SPECIES

A Thesis submitted for the degree of Doctor of Philosophy

School of Agriculture, Food and Wine

The University of Adelaide

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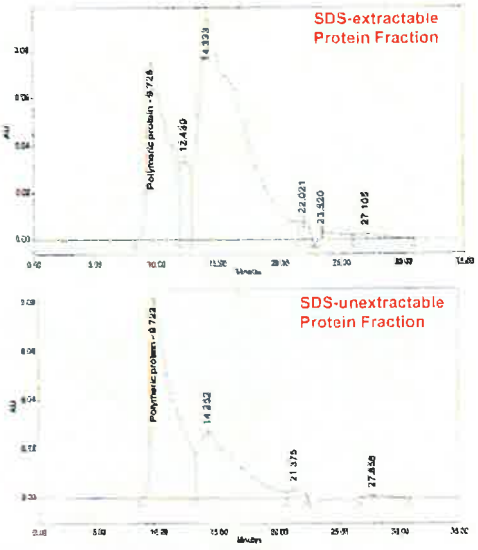
2002 Aroona Isoline Field Trial, Charlick, SA



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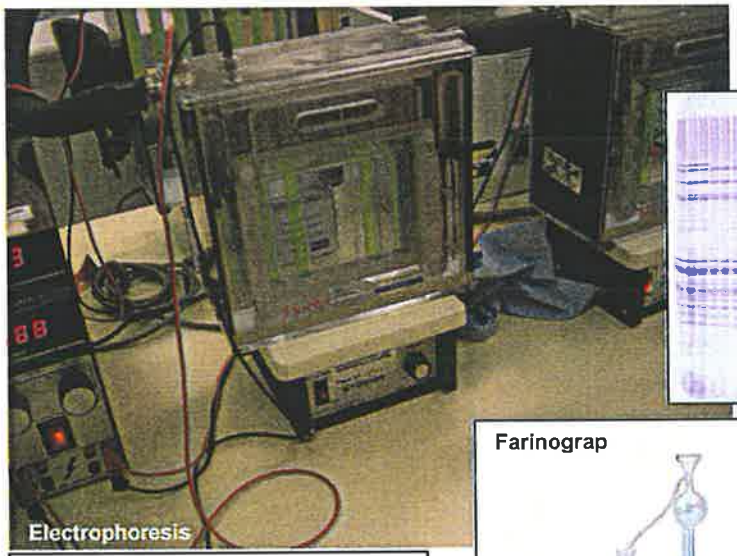

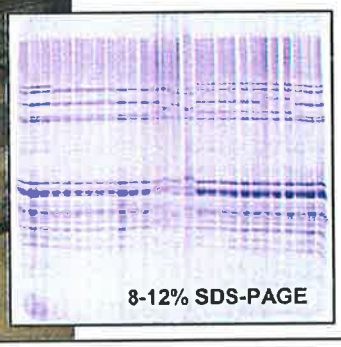

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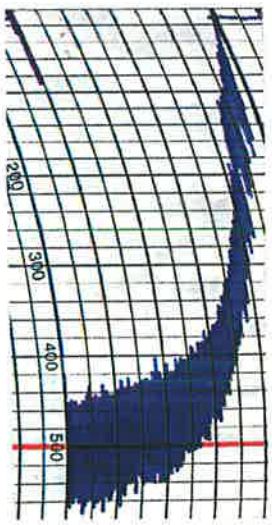

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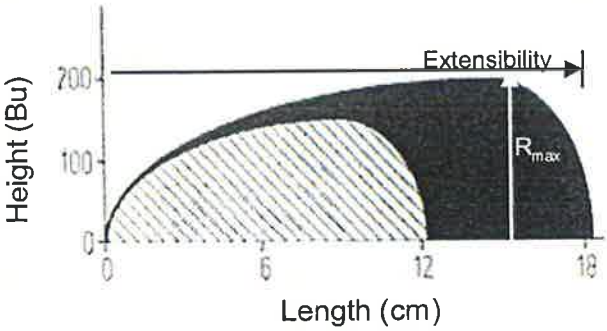

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28th June 2006

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ABSTRACT

Variation in quantity and quality of gluten proteins is largely responsible for the genotypic differences associated with the dough rheological parameters, maximum resistance (R_{max}) and extensibility (Ext.). In the context of bread making, doughs characteristic of good quality have moderate to high extensograph maximum resistance (R_{max}) and high extensibility (Ext.). The term usually applied to describe the balance between these two parameters is dough strength. Generally, weak doughs perform poorly in baking tests and as dough strength increases, bread making quality also increases. Important proteins that constitute the 'gluten complex' include high molecular weight glutenin subunits (HMW-GSs) and low molecular weight glutenin subunits (LMW-GSs). These proteins, which interact to produce large polymeric proteins, are coded at the *Glu-1* and *Glu-3* loci on group 1 chromosomes, respectively. Extensive allelic variation exists at each of the *Glu-1* and *Glu-3* loci. Field trials (4 years) and physical dough quality tests on harvested grain from a set of near-isogenic lines, differing in glutenin composition, were used to investigate the effect of numerous glutenin alleles on dough rheological parameters. Glutenin allele main effects were ranked as follows: *Glu-A1* $a = p = b > c$ for R_{max} and *Glu-A1* $a = b = p > c$ for Ext.; *Glu-B1* $i \geq b = c > d = a$ for R_{max} and *Glu-B1* $a = i = c \geq b \geq d$ for Ext.; *Glu-D1d* > *Glu-D1a* = *Glu-D1b* ≥ *Glu-D1f* for R_{max} and *Glu-D1* $a = b = f \geq d$ for Ext.; *Glu-A3* $d = b \geq c = f \geq a > e$ for R_{max} and *Glu-A3* $b = a = d = c = f \geq e$ for Ext.; *Glu-B3* $g \geq b = m \geq d = i = h = f \geq a \geq c$ for R_{max} and *Glu-B3* $i = d \geq g = f = m \geq b = c = h = a$ for Ext.; *Glu-D3 a-Gli-D1*$^{-}$ $= f \geq c = d = a \geq b$ for R_{max} and *Glu-D3* $d \geq$ *a-Gli-D1*$^{-}$ $\geq a \geq b = c = f$ for Ext. The influence of protein content and two-way glutenin allele interactions are also discussed.

Another aspect of this work investigated the relationship between HMW-GS expression

levels and quality. RP-HPLC was used to quantify the proportion (% area) of individual HMW-GSs relative to total HMW-GSs. Except for *Glu-B1d* (6+8*), the B-genome contributed the highest percentage of HMW-GSs and was significantly higher ($P<0.001$) in cultivars that contained the *Glu-B1al* allele. A high proportion of 1Bx subunits compared to 1Dx subunits (≈ 2.3, *Glu-B1al*) correlated with varieties reported to have extra strong dough properties, while a 1Bx:1Dx ratio of ≈ 1.3 (*Glu-B1 i, f, c, u* and *ak*) was typical of varieties with moderate to high dough strength characteristics. In varieties which contain *Glu-B1* alleles reported to produce weak doughs the 1Bx:1Dx value was ≈ 1.0 (*Glu-B1e*) and ≈ 0.6 (*Glu-B1d*). This suggests that the overall proportion of *Glu-B1* subunits has a major influence on dough strength and that the proportion of 1Bx relative to 1Dx subunits, as determined by RP-HPLC, could be used to predict dough quality. RP-HPLC analysis also enabled the identification of varieties that contained the *Glu-B1al* allele and over-expressed subunit *Glu-B1* 7x, including the most likely source of this allele in bread wheat cultivars.

Novel HMW-GS alleles in related wheat species with good quality potential were also identified. A simple small-scale screening assay was developed to efficiently assess the protein quality attributes associated with accessions of synthetic hexaploids, *T. tauschii* and *T. dicoccoides*. Development of the Turbidity assay is described and was used in conjunction with SE-HPLC and SDS-PAGE to confirm and characterise previously undescribed HMW-GSs. The HMW-GS composition of *T. dicoccoides* is discussed in detail where there were 49 HMW-GSs which combined to produce 54 different HMW-GS banding patterns. Accordingly, allelic designations were tentatively assigned to either individual or subunit pairs and these are also reported in this manuscript.

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LIST OF ABBREVIATIONS

AACC	American Association of Cereal Chemists
AGT	Australian Grain Technologies
A-PAGE	Acid – polyacrylamide gel electrophoresis
AH	Australian Hard
ANOVA	Analysis of variance
APH	Australian Prime Hard
APW	Australian Premium White
Area	Extensograph dough strength
Aril	Aroona recombinant inbred line
ASW	Australian Standard White
AWB	Australian Wheat Board Ltd.
AWCC	Australian Winter Cereal Collection
Bp	Base-pair
BU	Brabender units
CIMMYT	International Centre for Maize and Wheat Improvement
CWES	Canada Western Extra Strong
CSIRO	Australian Commonweatlth Scientific and Research Organization
DA	Double glutenin allele substitution of bread wheat cultivar, Aroona
dH_20	deionised water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
Ext.	Extensograph extensibility
FDBD	Farinograph dough breakdown
FDDT	Farinograph dough development time
FP	Flour protein
FWA	Farinograph water absorption
G	Alveograph swelling
GG	Grit gauge
GPC	Grain protein content
HCl	Hydrochloric acid
HMW-GS	High molecular weight glutenin subunit
HPLC	High performance liquid chromatography
HSE	Heat shock element
id	Internal diameter of HPLC column
IWIS	International Wheat Information System
kDa	Kilodaltons
kHz	Kilohertz
L	Alveograph extensibility
LMW-GS	Low molecular weight glutenin subunits
LV	Loaf volume
mA	Milliamps
MDDT	Mixograph dough development time
MPR	Mixograph maximum peak resistance
MRBD	Mixograph resistance breakdown
M_r	Molecular weight
mRNA	Messenger Ribonucleic acid
MWD	Molecular weight distribution

LIST OF ABBREVIATIONS (cont.)

N	Nitrogen
NaCl	Table salt
NIR	Near infra-red spectrophotometry
nm	Nanometer
NSW	New South Wales
NZ	New Zealand
P	Alveograph tenacity or maximum resistance
PCR	Polymerase chain reaction
PEB	Phosphate extraction buffer
PSI	Particle size index
PVDF	Polyvinylidine difluoride
REML	Residual maximum likelihood estimation
RFLP	Restriction fragment length polymorphism
R_m	Relative electrophoretic mobility
R_{max}	Extenosgraph maximum resistance
RP-HPLC	Reversed-phase high-performance liquid chromatography
SA	Single glutenin allele substitution of bread wheat cultivar, Aroona
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SED	Standard error of difference
SE-HPLC	Size-exclusion high-performance liquid chromatography
TA	Triple glutenin allele substitution of bread wheat cultivar, Aroona
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TP	Total protein as determined by SE-HPLC
%FPP	Percentage of polymeric protein in flour protein (SE-HPLC)
%PPP	Percentage of polymeric protein in total protein (SE-HPLC)
%UP	Percentage of SDS-unextractable protein in total protein (SE-HPLC)
%UPP	Percentage of SDS-unextractable polymeric protein in total polymeric protein (SE-HPLC)
%IP	Percentage of SDS-insoluble protein in total protein (Turbidity)
USA	United States of America
UV	Ultra violet
4-VP	4-vinylpyridine
W	Alveograph work input or gluten strength

CHAPTER 1

GENERAL INTRODUCTION

Wheat is the one of the most widely produced, consumed and traded cereal grains in the world today. In Australia, it is the most important commercial cereal crop grown, with wheat production for the period 1995-1999 averaging 20.5 million tonnes/year. Globally, this places Australia in tenth position as a wheat producer and in the top five wheat exporting countries (O'Brien *et al.*, 2001).

Wheat would not have attained its pre-eminent position in Australian agriculture without the endeavours of dedicated breeders and researchers. As early as 1892 collaboration between Mr W.J. Farrer (wheat breeder) and Professor F.B. Guthrie (cereal chemist), both of whom considered quality as important as yield and disease resistance in the development of new varieties, established a pattern of breeding and quality testing that is followed to the present day (Simmonds, 1989; McCarthy, 1993; Wrigley, 1993). An important contribution by Guthrie and Farrer to the developing science of plant breeding was to demonstrate that, for fixed lines, improvements to quality are maintained in subsequent generations (Simmonds, 1989; McCarthy, 1993).

"Grain quality" is a term used to define particular physiological attributes required for specific end-uses. The unique visco-elastic properties of wheat flour dough enables the manufacture of a diverse range of end products with the major use being for human consumption. Pan breads, flat breads, steamed breads, noodles, cakes, biscuits and pastry are examples of products produced from hexaploid wheat, also known as bread wheat or *Triticum aestivum*. Different end products require a different combination of quality attributes. The genes responsible for the fundamental aspects of quality are those which control grain hardness, protein content, protein quality, starch quality, lipids and enzymes.

In the context of bread making potential, dough strength is an important factor with both moderate to high resistance and high extensibility considered desirable (Pomeranz, 1988). The genes that govern dough strength are those which control protein content and glutenin composition. Although wheat varieties differ in their ability to accumulate protein, variation in protein content due to genotype is small compared to environmental growing conditions. In contrast, alleles that determine glutenin composition, explain a large proportion of the variation associated with loaf volume present in different cultivars with similar protein content (Simmonds, 1989).

Of considerable significance to the Australian wheat industry was the introduction of high-yielding semi-dwarf varieties from the International Centre for Maize and Wheat Improvement (CIMMYT, Mexico, D. F., Mexico) and the introgression of genes from wild wheat relatives to utilise novel disease resistance genes. While bread wheat has a relatively narrow genetic base, wild and primitive wheat relatives possess a high degree of genetic diversity making this germplasm a useful tool for increasing genetic variability and improving cultivated wheat (Sears, 1981). Already, foreign or 'alien' germplasm sourced from wild wheat relatives has provided valuable disease resistance genes to Australian breeding programs and as a result, numerous commercially important bread wheat cultivars have been released (O'Brien, *et al.*, 2001). The exploitation of wild wheat progenitors as a source of novel genes has primarily focused on disease and abiotic stress resistance, but could also be applied to identifying new glutenin alleles.

In order to gain significant advances in the bread making potential of Australian wheat, a thorough understanding of the effect different glutenin alleles on the rheological properties of wheat flour dough is required. Also, unexploited glutenin alleles, which confer exceptional dough quality attributes, could provide Australia with a market advantage over its competitors. As a way to understand glutenin effects and identify novel glutenin alleles,

the study reported in this thesis examines :

1) the effect of different glutenin alleles on dough rheological parameters.
2) the interaction between protein content and glutenin alleles to change dough rheology.
3) the relationship between expression levels of different glutenin subunits and their effects on dough strength.
4) an efficient screening method to predict protein quality on a small quantity of flour.
5) the genetic variation of glutenin alleles present in wild uncultivated wheat relatives with a view to improving bread making quality.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

If the Australian wheat industry is to remain competitive in the international market, it needs to be able to supply high quality grain suited to end-user requirements. Within a hardness class, protein composition becomes the primary basis for discriminating different end-uses, by virtue of its affect on dough rheological parameters (Pomeranz, 1990). Desirable gluten characteristics, especially for the production of pan breads, are moderate to high dough strength and high extensibility. Both dough strength and extensibility are influenced by the protein content and/or the composition of the wheat endosperm proteins. While protein content is determined mainly by the environmental growth conditions, the genes encoding the type of proteins present explain most of the varietal differences in bread making potential (Finney and Barmore, 1948).

Gluten proteins are encoded by large families of closely related genes present in all three genomes (ABD; 2n = 6x = 42) of modern hexaploid bread wheat. As a result, numerous combinations of gluten protein subunits exist in wheat cultivars. Although the relative proportions of the different types of gluten protein present are subject to environmental influence, research into gluten protein functionality has revealed that the highly heritable gluten proteins are responsible for the differences in dough strength among different wheat genotypes grown under the same environmental conditions (Payne *et al.*, 1987; Pomeranz, 1988; Panozzo and Eagles, 2000).

This project focuses on increasing the dough strength of Australian germplasm. Gluten proteins present in bread wheat and wild wheat progenitors have been investigated to

identify alleles that have the potential to improve protein quality. In this chapter a review of relevant published papers provides an overview of the fundamental aspects of grain quality, endosperm storage proteins, how they affect dough rheology and possible new sources of previously undescribed glutenin alleles that may enhance Australian wheat quality in relation to bread making.

2.2 ENDOSPERM STORAGE PROTEINS

The endosperm consists predominantly of starch and contains greater than 70% of the total grain protein. While some of the proteins fulfil metabolic or structural functions during endosperm development, most (over 80%) are storage proteins (Colot, 1990). These storage proteins, termed prolamins, posses a central domain made up of repeat motifs rich in proline and glutamine residues (Colot, 1990; Shewry *et al.*, 1992). Prolamins were first classified by Osborne in 1907 into two groups based on their solubility in aqueous alcohol (50% v/v). The gliadins are soluble in aqueous alcohol whereas the glutenins are insoluble, although native glutenin can be solubilised in 70% ethanol (Gupta and Shepherd, 1990).

2.2.1 Gliadins

Gliadins are monomeric proteins either because they do not contain any cysteine residues or because the cysteine residues present are involved in intermolecular disulfide bonds (Masci *et al.*, 2002). Gliadins consist of four subgroups, α, β, γ and ω identified by decreasing electrophoretic mobility in acid polyacrylamide gel electrophoresis (A-PAGE) (Jones *et al.*, 1959; Bietz *et al.*, 1977). The α-, β- and γ-gliadins have amino acid compositions similar to typical low molecular weight glutenin subunits, although their sequences differ significantly from the latter protein group (Masci *et al.*, 2002). These three gliadin types all have 'apparent' molecular weights in the approximate range of 30-45kDa, as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis

(SDS-PAGE) (Masci *et al.*, 2002). The ω-gliadins, sometimes termed "sulphur-poor", have higher 'apparent' molecular weights (44-74 kDa) on SDS-PAGE and distinctive amino acid compositions as they generally have no cysteines and at most one methionine (Shewry *et al.*, 1986; Tatham and Shewry, 1995; Hsia and Anderson 2001; Masci *et al.*, 2002).

2.2.2 Glutenins

The glutenin fraction contains monomeric subunits that slowly polymerise during grain development to form polymers with a range of molecular weights. This is achieved through intermolecular disulfide bonds via flanking cysteine residues of the glutenin protein subunits (MacRitchie, 1999). Polymeric glutenin when reduced by mercaptoethanol or equivalent agent produces two types of monomeric proteins, high molecular weight glutenin subunits (HMW-GSs) and low molecular weight glutenin subunits (LMW-GSs) (Colot, 1990). These glutenin subunits are further classified into four subgroups according to their mobility in SDS Gradient PAGE, namely the A-, B-, C- and D-regions of electrophoretic mobility (Figure 2-1).

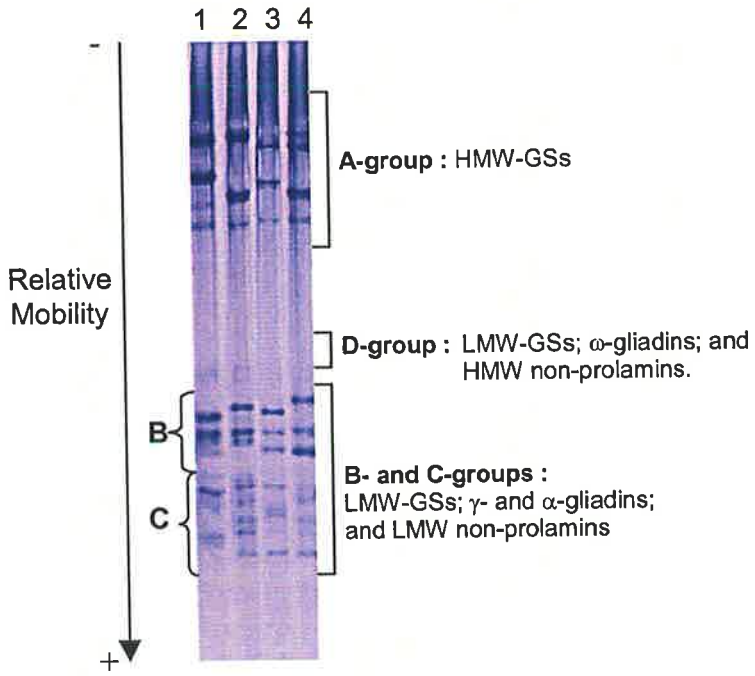


Figure 2-1 SDS Gradient PAGE (8-12%) separation of glutenin subunits of standard hexaploid wheat cultivars indicating regions of electrophoretic mobility and associated subunits. Lane 1 - Chinese Spring; Lane 2 - Gabo; Lane 3 - Jabiru; and Lane 4 - Wilgoyne (Gupta and Shepherd, 1990; Gianibelli *et al.*, 2001)

The A-group corresponds to the HMW-GSs, with an 'apparent' molecular weight range of 80 kDa to 130 kDa on SDS-PAGE, or between 60 and 90kDa based on derived amino acid sequences. The B- (42-51 kDa) and C- (30-40 kDa) groups contain the predominant LMW-GSs (≈60% of total glutenin), many of which, especially in the C-group, are distantly related to γ- and α-gliadins, while the LMW-GSs present in the D-region of electrophoretic mobility (≈ 58 kDa) are highly acidic and related to ω-gliadins (Gianibelli *et al.*, 2001)

2.2.3 Chromosomal location of genes encoding prolamins

2.2.3.1 High molecular weight glutenin subunits

The genes that encode HMW-GSs (A-subunits) are located at the *Glu-1* loci on the long arm of group 1 chromosomes, 1AL, 1BL and 1DL, loci *Glu-A1*, *Glu-B1* and *Glu-D1* respectively. Each *Glu-1* locus consists of two closely linked genes that encode two types of HMW-GSs (x- and y-type). The x-type HMW-GSs have a slower electrophoretic mobility in SDS-PAGE and higher molecular weight than y-type HMW-GSs (Bietz *et al.*, 1975; Lawrence and Shepherd, 1980, 1981a; Payne and Lawrence, 1983).

Theoretically, a single hexaploid wheat variety should contain six different HMW-GSs but because some of these genes are not expressed they usually possess 4 to 5 HMW-GSs and some as few as 3 (Gianibelli *et al.*, 2001). Expression of *Glu-A1* y-type subunits is almost always silent in hexaploid wheat with only a few exceptions reported in the literature (Johansson *et al.*, 1993; Margiotta *et al.*, 1996). Sometimes genes encoding *Glu-A1* x-type subunits are not expressed while silencing of *Glu-B1* y-type subunits occurs less often. Interestingly, expression of both *Glu-A1* x- and y-type subunits is not unusual in A-genome diploid and tetraploid (AABB) wild wheat progenitors (Shewry *et al.*, 1992; Gianibelli *et al.*, 2001).

2.2.3.2 Low molecular weight glutenin subunits

Most of the B-group LMW-GSs and some of the C-group LMW-GSs are controlled by genes at the *Glu-A3*, *Glu-B3* and *Glu-D3* loci on chromosomes 1AS, 1BS and 1DS, respectively (Jackson *et al.*, 1983; Masci *et al.*, 2002). The multigene *Glu-3* loci encode numerous closely linked genes (Gupta and Shepherd, 1988). Early results based on protein gel electrophoresis indicated that a single hexaploid wheat variety could contain 7-16 LMW-GSs (Payne, 1987; Colot, 1990). However, N-terminal sequencing of protein subunits and Southern blot analysis using DNA identified between 30 to 40 different LMW-GSs in a single variety (Lew *et al.*, 1992; Cassidy *et al.*, 1998; Cloutier *et al.*, 2001). Chromosome 1AS encodes the fewest LMW-GSs with some cultivars not expressing any LMW-GSs coded by the *Glu-A3* locus whereas chromosome 1BS exhibits extensive polymorphism (Gupta and Shepherd, 1990).

2.2.3.3 Gliadins

The multigene loci that encode gliadins are similar to the *Glu-3* loci, containing closely linked genes that are inherited together, and are often referred to by researchers as 'gliadin blocks' (Doekes, 1973). The *Gli-1* loci, which encodes ω-, γ- and a few β-gliadins are located on the short arms of group 1 chromosomes, within close proximity to the *Glu-3* loci (Payne *et al.*, 1982). Evidence of little or no recombination between *Glu-3/Gli-1* indicates that these loci are closely linked with the distance between *Glu-B3* and *Gli-B1* estimated to be 1.7 cM (Singh and Shepherd, 1988). The *Gli-1* loci encode between 3-9 genes of each of the γ- and ω-gliadins and the genes encoding γ-gliadins are distal to those encoding ω-gliadins with respect to the centromere (Payne, 1987; Colot, 1990; Pogna *et al.*, 1990). Kasarda *et al.* (1983) used N-terminal protein sequences to classify gliadins based on the first three amino acids. The genes encoding ARQ-/ARE-type ω-gliadins are most likely located on 1AS and 1DS chromosomes (Tatham and Shewry, 1995; Masci *et al.*, 1999; Hsia and Anderson, 2001) while genes that control SRL-types (γ-gliadins) map

to short arm on chromosome 1B (Tatham and Shewry, 1995; Dupont *et al.*, 2000; Hsia and Anderson, 2001).

The structure and location of the *Gli-2* loci is less well documented but have been mapped to the distal portion of the short arms on chromosomes 6A, 6B and 6D (Dvorak and Chen, 1984; Payne *et al.*, 1984b; Metakovsky, 1991). Gliadin blocks encoded by *Gli-2* are mainly associated with α- and β-gliadins, but also code for some γ-gliadins. In a single hexaploid wheat variety there are approximately 15-20 α- and β-gliadins (Payne, 1987; Colot, 1990).

2.2.3.4 Additional minor loci

Subunits associated with the D-region of electrophoretic mobility, although considered to be LMW-GSs are modified ω-gliadins (Gianibelli *et al.*, 2001). While typical ω-gliadins do not contain any cysteine residues these modified ω-gliadins contain a single cysteine codon in their sequence enabling them to form intermolecular disulfide bonds and become part of the polymeric fraction (Masci *et al.*, 1993, 1999 and 2002). Genes encoding ω-type LMW-GSs have been mapped halfway between *Glu-1* and *Gli-1* on chromosomes 1A and 1B at *Gli-A3* and *Gli-B3* loci, respectively (Galili and Feldman, 1984; Sobko, 1984; Jackson *et al.*, 1985; Metakovsky *et al.*, 1986; Payne *et al.*, 1988; Dubcovsky *et al.*, 1997). On chromosome 1D, genes encoding ω-type LMW-GSs are completely linked to the *Gli-D1* locus (Payne *et al.*, 1986; Pogna *et al.*, 1995; Dubcovsky *et al.*, 1997).

In addition to a small number of typical LMW-GSs, the majority of C-group glutenin subunits also have gliadin-like sequences (Tao and Kasarda, 1989; Lew *et al.*, 1992; Masci *et al.*, 1995). These gliadin-like glutenin subunits appear to be mutated forms of α- and γ-gliadins with an odd number of cysteine residues rather than the usual complement of six and eight, respectively (D'Ovidio *et al.*, 1995; Sreeramulu and Singh, 1997; Anderson and

Greene, 1997; Masci *et al.*, 2002). These gliadin-like C-group LMW-GSs are coded on chromosome groups 1 and 6, probably by unidentified genes located on loci tightly linked to the *Gli-1* and *Gli-2* loci or by genes located within the *Gli* loci (Masci *et al.*, 2002).

Two C-group subunits have been reported with 'apparent' molecular weights of 30kDa and 32kDa using SDS-PAGE. The loci which encode these LMW-GSs have been designated *Glu-D4* (1D) and *Glu-D5* (7D), respectively (Sreeramulu and Singh, 1997). Their exact localisation on the chromosomes has not yet been established (Sreeramulu and Singh, 1997; Gianibelli *et al.*, 2001). *Glu-B2* encodes a B-group LMW-GS that is genetically linked to the ω-type glutenin genes at the *Gli-B3* locus in the tetraploid wheat species, *T. turgidum* ssp. *durum* and *T. turgidum* ssp. *dicoccoides* (Ruiz and Carrillo, 1993; Liu, 1995; Liu and Shepherd, 1995; Dubcovsky *et al.*, 1997; Nieto-Taladriz *et al.*, 1997). Even though the *Glu-B2* locus is present in hexaploid wheat (RFLP marker, *Xbcd249*) and located in a similar position as for tetraploid wheat, an expressed protein subunit has not been indentified using SDS-PAGE (Dubcovsky *et al.*, 1997).

2.2.4 Nomenclature and identification

In order to assign allelic designations and identifiers to gene products, a considerable appreciation of the genetics involved must be obtained. The pioneering work conducted by a large number of researchers (Shepherd, 1988) has been instrumental in our understanding and the characterisation of glutenin alleles.

2.2.4.1 HMW-GSs of cultivated polyploid wheat

A significant development in the identification of HMW-GS alleles was the independent introduction by both Payne in Cambridge and Lawrence in Adelaide (Shepherd, 1988), of the Laemmli (1970) method of SDS-PAGE. This method improved the resolution of reduced HMW-GSs facilitating the identification of electrophoretically different HMW-GSs in a wide range of hexaploid wheat cultivars, resulting in allelic designations for the

various HMW-GSs that make up *Glu-1* gene complexes (Shepherd, 1988). The numbering system for HMW-GSs devised by Payne and Lawrence (1983) assigns allelic designations to HMW-GS pairs encoded on the same chromosome (Figure 2-2).

Other HMW-GS pairs not depicted in Figure 2-2 but relevant to this work include the *Glu-B1* subunits that have similar electrophoretic mobilities. In addition to *Glu-B1b* (7+8), a further three *Glu-B1* alleles, namely *Glu-B1u* (7*+8), *Glu-B1ak* (7*+8*) and *Glu-B1al* (7+8*), have now been identified which cannot be easily differentiated from each other using SDS-PAGE (Marchylo *et al.*, 1992). Although discrimination between the *Glu-B1* subunits 1By8 and 1By8* can be achieved using reversed-phase high-performance-liquid-chromatography (RP-HPLC), the problem of identification lies with an inability to routinely differentiate between the HMW-GSs 1Bx7 and 1Bx7* with either SDS-PAGE or RP-HPLC. However, optimisation of SDS-PAGE specifically to resolve 1Bx7* from 1Bx7, has revealed that subunit 1Bx7* is slightly more mobile than 1Bx7 (Marchylo *et al.*, 1992). Fortunately, molecular techniques have recently been developed that enable genotypic identification of HMW-GSs 1Bx7, 1Bx7* and 1Bx17 (Butow *et al.*, 2003).

The general consensus is that *Glu-B1b* is rare and refers to the *Glu-B1* subunits present in Chinese Spring. Australian varieties that contain similar HMW-GSs are routinely assigned the allelic designation, *Glu-B1u* (McIntosh *et al.*, 1998; Zhen, 1993). When subunit *Glu-B1* 8*y is present, subunit 1Bx7 is typically over-expressed (7^{OE}+8*) and has the allelic designation *Glu-B1al*. However, HMW-GS 1By8* is not always associated with over-expression and this is indicative of the presence of *Glu-B1ak* (Marchylo, *et al.*, 1992). Interestingly, a survey of 107 Argentinian bread wheat cultivars, a country renowned for producing varieties with exceptional bread making quality, showed that the *Glu-B1u* allele was not present and that some cultivars that had the *Glu-B1al* allele did not over-express subunit 1Bx7 (Gianibelli *et al.*, 2002).

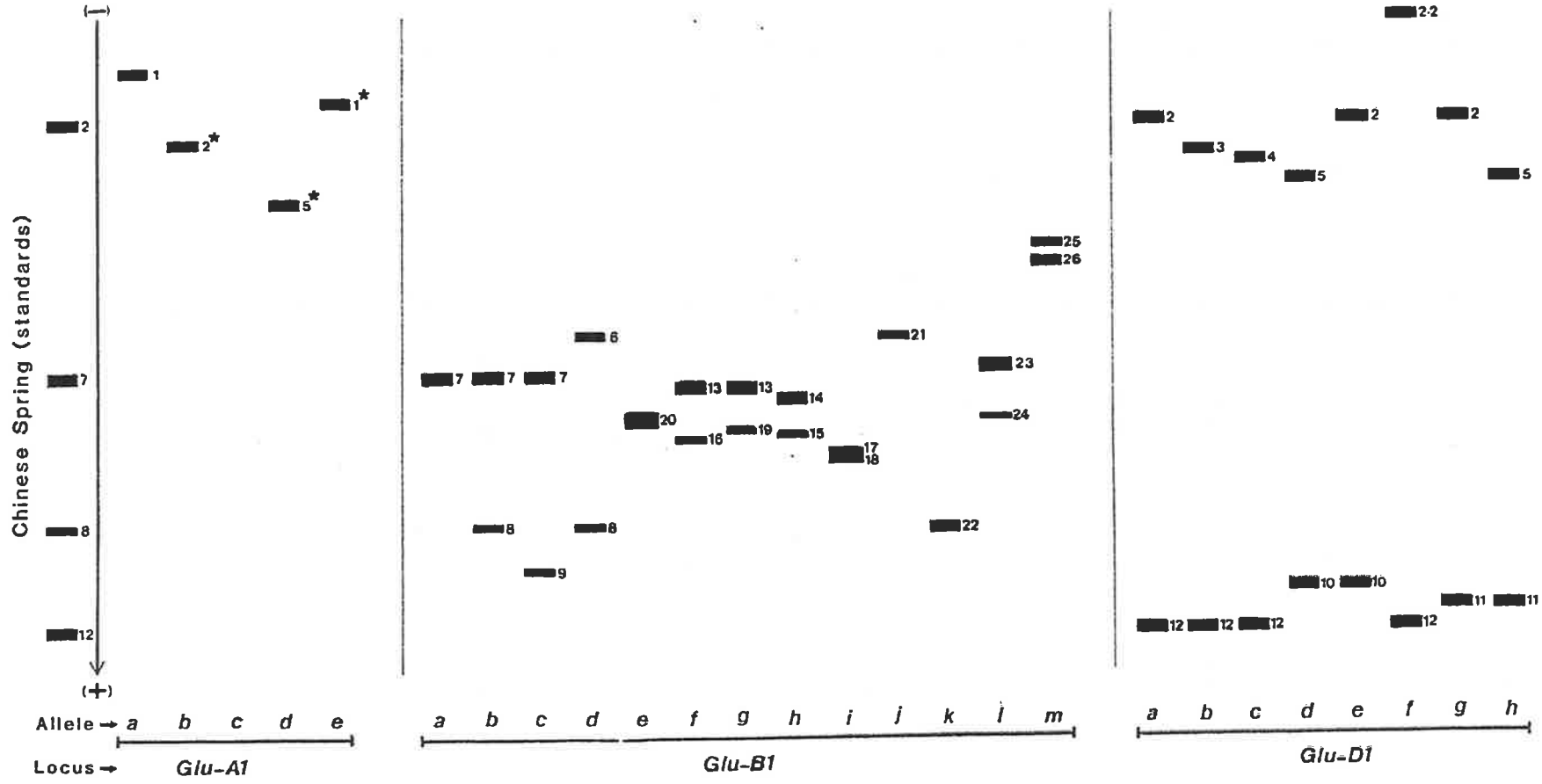


Figure 2-2 Simplified schematic showing the numbering system developed by Payne and Lawrence (1983) of various HMW-GSs and their allelic designations typically found in hexaploid wheat.

For *Glu-B1c*, subunit 1By9 is always present with 1Bx7* and not 1Bx7 as previously thought (Marchylo *et al.*, 1992; Gianibelli *et al.*, 2002). Similarly, the standard variety for the *Glu-B1d* (6+8) allele, Hope, contains a *Glu-B1* y-type subunit that more closely resembles 1By8* than 1By8 (McIntosh *et al.*, 1998; Vawser and Cornish, 2004). This has also been observed in other varieties (Sutton, 1991). In light of this evidence, the HMW-GSs associated with the allelic designations *Glu-B1c* and *Glu-B1d* should be changed to 1Bx7* + 1By9 and 1Bx6 + 1By8*, respectively. Also of note is that *Glu-B1e*, also known as HMW-GS 1Bx20, has 2 subunits, 1Bx20 + 1By20 (Tatham *et al.*, 1991; Zhen, 1993).

2.2.4.2 HMW-GSs alleles in wild wheat progenitors

The restricted number of hybridisations in the evolution of hexaploid wheat means that the pool of potentially useful genes available to wheat breeders is limited compared to that present in the uncultivated ancestral species (Sears, 1981; Hoisington *et al.*, 1999). Fortunately, bread wheat has a large assortment of relatives diverse in phenotype and adaptation. These bread wheat progenitors are highly polymorphic for seed-storage proteins and an important source of new glutenin genes for the improvement of bread making quality. As a precedent, important disease resistance genes from wild wheat progenitors have been utilised in the development of commercially important bread wheat cultivars.

The wild species of the subtribe *Triticinae* show varying degrees of cytogenetic affinity and phylogenetic relationships between themselves and cultivated bread and durum wheat. They can be classified into two main groups, depending on whether their genomes are homologous (group 1) or homoeologous (group 2) to those of cultivated wheat (Table 2-1). The reputed diploid donors of the A-genome (*T. monococcum* ssp. *monococcum* and *boeoticum* and *T. urartu*) and the D-genome (*T. tauschii*) to cultivated wheat and polyploids sharing one or two genomes with cultivated wheat are included in the first

Type of gene pool			Species and genomic formulae
1	Species with homologues of wheat genome		
	(a) The tetraploid progenitor		*T. turgidum* ssp. *dicoccoides* (AB)
	(b) The diploid donors of the A- and the D-genomes		*T. monococcum* ssp. *monoccum* and *boeticum* or *T. urartu* (A) *T. tauschii* (D)
	(c) Polyploid with one homologous genome		
		(i) The A-genome	*T. timopheevii* ssp. *timopheevii* and *araraticum* (AG)
		(ii) The D-genome	*T. crassum* (DM^{cr}, DD_2M^{cr}) *T. ventricosum* (DM^{v}) *T. cylindricum* (CD) *T. juvenile* ($DM^{cr}U$) *T. syriacum* ($DM^{cr}S$)
2	Species with homoeologous genomes		
	(a) Closely related species		*T. searsii* (S^{s}) *T. longissimum* (S^{l}) *T. sharonensis* (S^{l}) *T. bicorne* (S^{b}) *T. speltoides* (S) *T. variabile* (US^{v}) *T. kotschyi* (US^{v})
	(b) Less closely related species		*T. tripsacoides* (M^{t}) *T. dichasians* (C) *T. comosum* (M) *T. umbellulatum* (U) *T. uniaristatum* (M^{u}) Other U-containing polyploids Several *Agropyron* species
	(c) Distantly related species		Species of *Secale*, *Haynaldia*; numerous species of *Agropyron* and of other genera of the *Triticinae* and *Hordeinae*

Table 2-1 Groups of wild relatives of wheat, listed in decreasing order of their presumed closeness of relationship to bread wheat (*T. aestivum*, ABD) (Sears, 1981)

group. The second group of wild relatives includes species that are more distantly related to cultivated wheat. The first group, which logically has been the focus of most research, crosses readily with cultivated wheat without recourse to special methodologies, while for the second group, the transfer of genetic material can be achieved only by inducing homoeologous pairing and crossing-over or translocations (Sears, 1981; Lafiandra *et al.*, 1993b).

Analysis of HMW-GS variability has been conducted in a few species, including the diploid donor species of the bread wheat genomes. Waines and Payne (1987) and Ciaffi *et al.* (1992) studied Einkorn wheats (A-genome), Lagudah and Halloran (1988) investigated goat grasses (D-genome), and Fernandez-Calvin and Orellana (1990) and Urbano *et al.* (1993) the *Sitopsis* section (likely donors of the B-genome). Other genomes present in the *Triticeae* tribe have also been studied and include *Secale cereale* (1R), *Hordeum valgare* (5H), *T. umbellulata* (1U), *T. comosum* (1M), *T. markgrafi* (1C) and *Agropyrum elongatum* (1E) (Lawrence and Shepherd, 1981b; Law and Payne, 1983; and Rodriguez-Quijano *et al.*, 2001).

2.2.4.2.1 D-genome

The diploid donor of the D-genome of bread wheat (*T. aestivum* ssp *aestivum*) and other hexaploid variants (ssp. *spelta*, *vavilovii*, *macha*, *compactum* and *sphaerococcum*) is generally accepted to be *T. tauschii* (*Aegilops squarrosa*) (Kihara, 1944; McFadden and Sears, 1946; Riley, 1965). There are two recognised subspecies of *T. tauschii*, ssp. *eusquarrosa* and *strangulata*. Varietal classes in the former subspecies are var. *typical*, *meyeri* and *anathera*, while var. *strangulata* is the only member of the latter subspecies (Lagudah and Halloran, 1988).

Lagudah and Halloran (1988) studied the variation in HMW-GS composition of 79

T. tauschii accessions. SDS-PAGE analysis of total protein identified 14 HMW-GS combinations (*Glu-D^{t}1*) and more have been reported in subsequent studies (Figure 2-3). In a study of 198 accessions, Yan *et al.* (2003) identified 42 HMW-GS banding patterns occurring at various frequencies, involving 9 x-type (2.1^t, 1.5^t, 1.5*^{t}, 2^t, 3^t, 4^t, 5.1^t, 5^t and 5*^{t}) and 13 y-type (10^t, 10.1^t, 10.2^t, 10.3*^{t}, 10.4^t, 11^t, 12^t, 12.1*^{t}, 12.2*^{t}, T$_2^t$, 12.3^t, 12.4^t and 12.5^t) subunits. Of the 42 subunit combinations, 3^t+12^t (15.2%), 2^t+10^t (12.6%), 4^t+12^t (9.6%), 5^t+10^t (8.6%) and 2^t+12^t (6.6%) were the most frequent. A similar frequency had previously been reported by Gianibelli *et al.* (1998) for subunits 2^t+10^t, which accounted for 15.6% of 173 accessions possessing 40 different HMW-GS combinations in that study.

Not shown in Figure 2-3 are subunits *Glu-D^{t}1* 4^{t}x, 5*^{t}x, and 12^{t}y. Subunit 1Dx4^t has a mobility between subunits 1Dx3^t and 1Dx5.1*^{t}, subunit 1Dx5*^{t} is slower than subunit 1Dx5^t and faster than subunit 1Dx4^t while the *Glu-D^{t}1* 12y^t subunit has a similar electrophoretic mobility to subunit *Glu-D1* 12y in bread wheat. With the exception of the largest HMW-GS present in bread wheat, *Glu-D1* 2.2x, variation based on SDS-PAGE mobilities reported by Lagudah and Halloran (1988), Gianibelli *et al.* (1998) and Yan *et al.* (2003) for *T. tauschii* encompassed the analogous *Glu-D1* subunits present in hexaploid wheat species, including those in bread wheat. However, even though the electrophoretic mobilities of subunits across species were similar, DNA sequence comparisons revealed that they are not the same. Subunit 1Dy12^t from *T. tauschii* has greater homology to subunit 1Dy10 than to subunit 1Dy12 of bread wheat (Mackie *et al.*, 1996b) and subunit 1Dx5^t does not contain the extra cysteine residue present in the bread wheat 1Dx5 subunit (Mackie *et al.*, 1996a).

The *Glu-D1*yT$_2^t$ subunit had been reported sometimes to be present with another y-type glutenin, termed T$_1^t$ (Lagudah and Halloran, 1988) however, biochemical and genetic

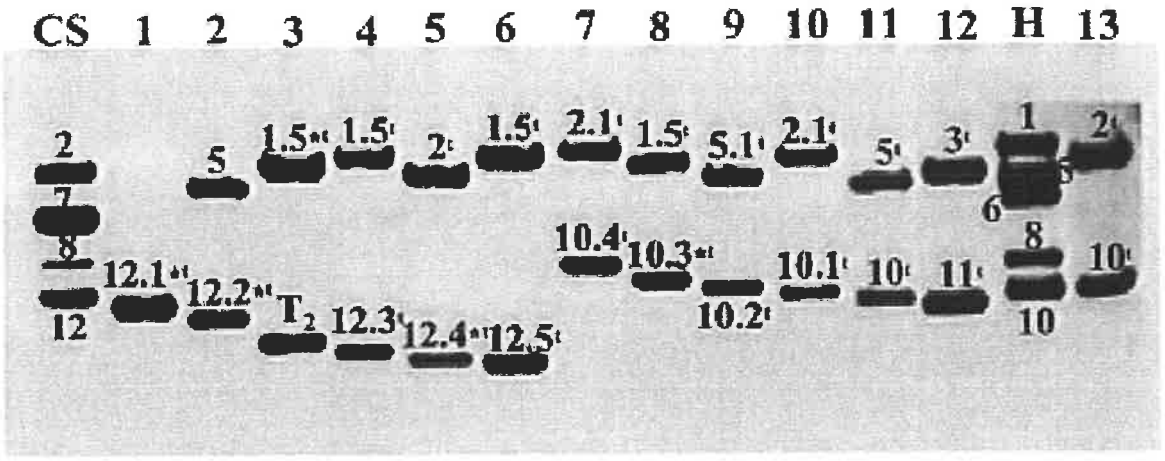


Figure 2-3 HMW-GS compostion of 13 *T. tauschii* accessions (1-13) by SDS-PAGE (10%). Two bread wheat lines Chinese Spring (CS) and Hope (H) were used as standards for HMW-GS identification (Yan *et al.*, 2003).

analyses recently showed that the T_1^t subunit is a monomeric ω-gliadin encoded at a new locus, *Gli-DT1* on the short arm of chromosome 1D (Mackie *et al.*, 1996a; Gianibelli *et al.*, 2002; Yan *et al.*, 2003). A single *T. tauchii* accession null for x-type and two others which were null for y-type subunits have also been identified (Gianibelli *et al.*, 2001; Yan *et al.*, 2003).

The production of synthetic hexaploids by colchicine treatment of durum x *T. tauschii* hybrid seedlings has proven to be an excellent mechanism for the exploitation of genetic variability present in the latter diploid species. In 1996, 430 synthetic hexaploids encompassing 250 *T. tauschii* accessions were produced with a view to incorporate the entire CIMMYT collection (490) of *T. tauschii* accessions into a hexaploid background (Mujeeb-Kazi *et al.*, 1996). A significant advantage of using synthetic hexaploids is that desirable genes can easily be introgressed into bread wheat hence enabling the effects of incorporated genes to be evaluated in a cultivated wheat background. At CIMMYT, breeders have extensively crossed synthetics with bread wheat and several of CIMMYT's advanced bread wheat lines carry genes from *T. tauschii*, although these are mainly for disease resistance and agronomic traits (Villareal *et al.*, 1996; Pena *et al.*, 1996).

2.2.4.2.2 A-genome

The study by Ciaffi *et al.* (1992) of diploid wild wheat possessing the A-genome (138 accessions of *T. urartu* and 92 accessions of *T. boeoticum*) identified 22 alleles for the *Glu-A1* locus – 9 in *T. urartu* and 13 in *T. boeoticum*. The HMW-GSs in most *T. boeoticum* accessions presented a major x-type subunit with similar electrophoretic mobilities to that of 1Ax and 1Dx subunits of bread wheat, and a series of less prominent y-type subunits of faster mobility, although one was usually more dominant. In this case, the mobility of the dominant y-type subunits was intermediate between 1Bx and 1Dy subunits of bread wheat. However, some accessions had only two well-distinguished y-type subunits where the mobility of the fastest y-type subunit was intermediate between those of 1Bx and 1By subunits of cultivated wheats (Figure 2-4). No *T. boeoticum* accession possessed only a single major component (Lafiandra *et al.*, 1993b).

The HMW-GS composition of *T. urartu*, exhibiting one major 1Ax and 1Ay subunit, was quite different from that present in *T. boeoticum* (Waines and Payne, 1987). No accessions showed the multitude of minor Ay subunits which were characteristic of *T. boeoticum*. The range of SDS-PAGE mobilities of *T. urartu* 1Ax subunits was similar to those of *T. boeoticum*, whereas the y-type subunits had electrophoretic mobilities greater than the dominant 1Ay subunits detected in *T. boeoticum* (Figure 2-4) (Lafiandra *et al.*, 1993b). Some *Glu-1* genes are known to be inactive in different *Triticum* species (Galili *et al.*, 1988). This is also the case for *T. urartu*. The 1Ay subunit was not expressed in 15% of the 138 *T. urartu* accessions analysed by Ciaffi *et al.* (1992), while, contrary to the findings of Waines and Payne (1987) and Galili *et al.* (1988), a moderate degree of gene inactivity (4%) and varying levels of expression was also associated with 1Ax subunits (Lafiandra *et al.*, 1993b).

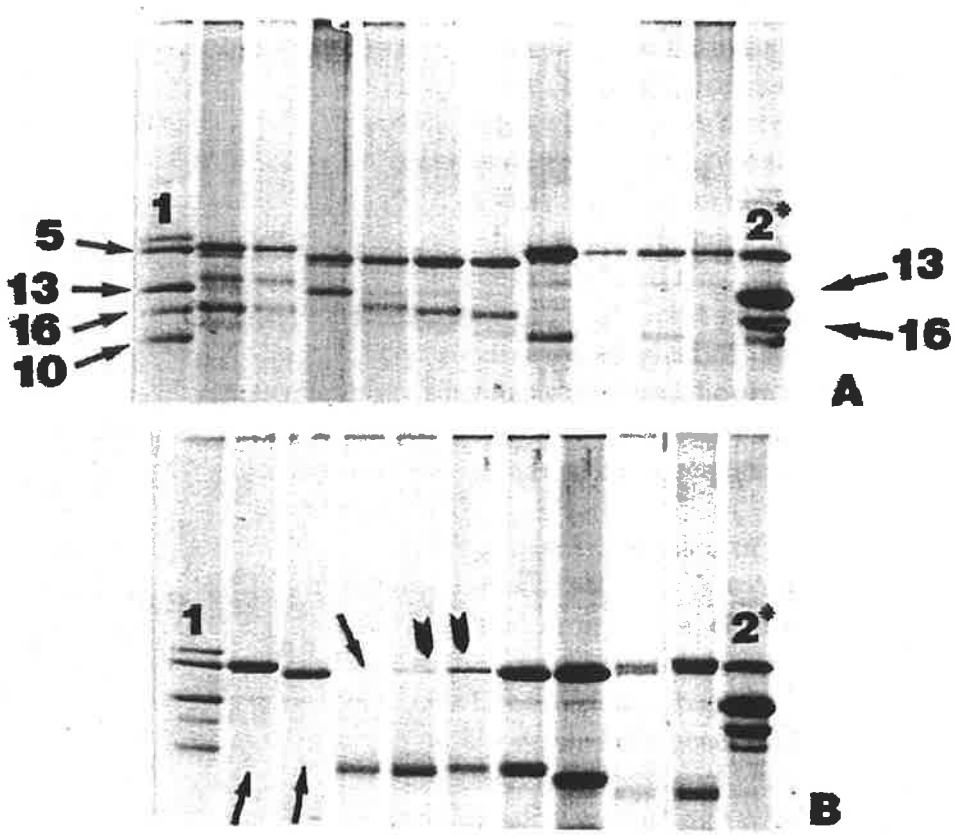


Figure 2-4 SDS-PAGE separation of HMW-GSs from accessions of a) *T. boeoticum* and b) *T. urartu* compared with those of a bread and a durum wheat cultivar. Small arrows indicate the absence of HMW glutenin x- or y-type subunits in *T. urartu*. Large arrows indicate different levels of staining density (Lafiandra *et al.*, 1993b).

T. monococcum, *T. boeoticum* and *T. urartu* possess the A-genome present in polyploid wheats. SDS-PAGE of the HMW-GSs of these diploid species showed that *T. monococcum* ssp. *monococcum* and *boeoticum* had similar banding patterns, whereas the banding patterns of equivalent proteins in *T. urartu* were quite distinct and similar to those of *T. dicoccoides* and *T. aestivum* (Caldwell and Kasarda, 1978; Konorev *et al.*, 1979; Kerby and Kuspina, 1987; Waines and Payne, 1987; Dvorak *et al.*, 1988). This indicates that the A-genome of *T. monococcum* is typical of the *T. timopheevi* (AG) group whereas the A-genome of *T. urartu* is more closely related to the *T. turgidum* (AB) group (Lafiandra *et al.*, 1993b).

2.2.4.2.3 A- and B-genomes

The donor of the B-genome has not been conclusively established but it is generally accepted that it was a diploid member of the *Sitopsis* section of the genus *Aegilops* (this genus is now commonly included in the *Triticum* genus). *T. searsii, T. longissima, T. speltoides* and *T. sharonensis* have all been reported as possible donors of the B-genome to cultivated polyploid wheats (Urbano *et al.*, 1993; Simmonds, 1989). These putative cultivated wheat progenitors possess the homoeologous S-genome which theoretically could be exploited to increase the range of variation of glutenin subunits in cultivated wheats (Moonen and Zeven, 1985). This approach is, however, difficult because of the reduced homology between the cultivated wheat and progenitor chromosomes.

Alternatively, chromosomes of the wild tetraploid progenitor, *T. dicoccoides* (2n = 4x = 28; genomes AABB), which possess greater allelic variation at both *Glu-A1* and *Glu-B1* loci than bread wheat, show regular pairing with both the A- and B-genomes of cultivated durum and bread wheat (Levy *et al.*, 1985; Levy *et al.*, 1988; Nevo and Payne, 1987; Levy and Feldman, 1988; Ciaffi *et al.*, 1993). When comparing HMW-GS mobilities with those in bread and durum wheat, Payne and Lawrence (1983) identified 27 new allelic variants in *T. dicoccoides*, 12 at the *Glu-A1* locus and 15 at the *Glu-B1* locus. Of the new variants, 6 alleles at the *Glu-A1* locus had both x- and y-type subunits (Figure 2-5). As expected, the electrophoretic mobilities of 1Ay subunits in *T. dicoccoides* were very similar to *T. urartu*, and no accessions had the multitude of minor 1Ay subunits characteristic of *T. boeoticum* (Lafiandra *et al.*, 1993b).

Clearly, the potential for utilizing genes from wild wheat relatives to improve both bread and durum wheat quality is large. A considerable global research effort focused on screening and characterising alien germplasm for desirable genes is currently being undertaken. However, since the early 1990's little has been published and available in the

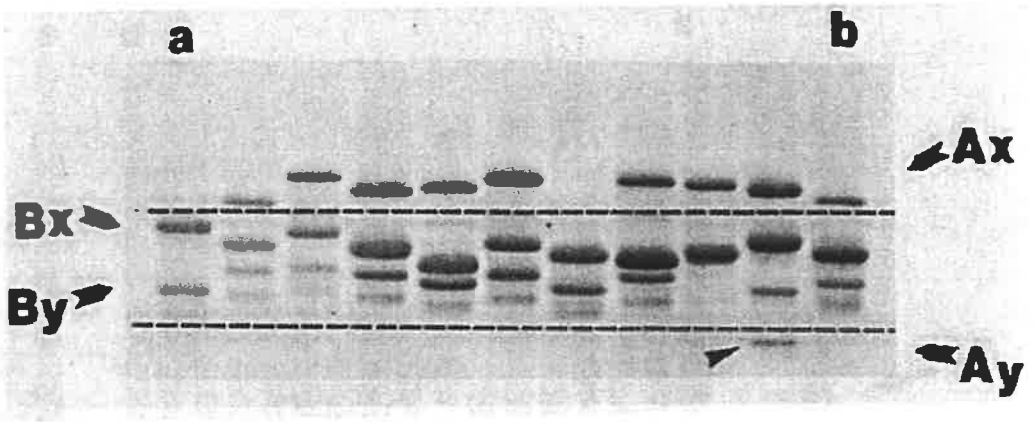


Figure 2-5 SDS-PAGE separation of HMW-GSs from *T. dicoccoides* compared with those of two durum wheat cultivars (a and b). The broken lines mark the area of Bx and By subunits. An example of material possessing both Ax and Ay subunits is indicated by a small arrow (Lafiandra *et al.*, 1993b)

public domain, particularly in relation to *T. dicoccoides*. It appears that nothing has been published specifically identifying the glutenin subunits present in *T. dicoccoides* aimed at assigning allelic designations. Further, the majority of papers reporting the genetic variation of glutenin subunits in *T. tauschii* do not provide the information required to identify accessions. As a consequence, standard *T. tauschii* accessions that could be used to assist HMW-GS identification on SDS-PAGE have not been implemented here.

A large proportion of this study was dedicated to screening exotic germplasm, namely synthetic hexaploids, *T. tauschii* and *T. dicoccoides* accessions for favourable protein characteristics, which once identified, were futher characterised using SDS-PAGE. To do this efficiently, a simple Turbidity assay was developed as an initial screening method and used in conjunction with SE-HPLC to determine the amount of specific protein fractions present in the germplasm being investigated. This information was then used to evaluate and select accessions considered to possess high protein quality attributes and that possibly contained previously undescribed HMW-GS alleles that may, when introgressed into well-adapted germplasm, improve the bread making quality of Australian bread wheat. Many of the different HMW-GSs present in *T. dicoccoides* accessions were identified and allelic

designations have been assigned to individual subunits or subunit pairs to facilitate their selection in future generations.

2.2.4.3 LMW-GSs of cultivated polyploid wheat

Allelic designations for LMW-GSs have been assigned to clusters of genes encoded at the same loci, a difficult task because of the complex nature of the *Glu-3* and other minor loci. Advances in the characterisation of LMW-GS alleles, which led to an unprecedented number of new allelic variants being described for LMW-GSs, was achieved by the production of wheat-rye substitution lines of group 1 chromosomes by Gupta and Shepherd (1990). These lines simplified electrophoretic patterns and allowed closer study of individual LMW-GS alleles and identified 20 different banding patterns (LMW-GS blocks) in a collection of 222 hexaploid wheats from 32 countries (Gupta and Shepherd, 1988 and 1990). Figure 2-6 depicts the LMW-GS allelic variants observed by Gupta and Shepherd (1990) showing the 6, 9 and 5 allelic variants identified for *Glu-A3*, *Glu-B3* and *Glu-D3*, respectively.

Even though the electrophoretic banding patterns associated with many LMW-GS alleles has been determined, reliable routine identification can still prove problematic. To assist in the identification of LMW-GSs, the close genetic linkage between the *Glu-3* and *Gli-1* loci is often exploited (Singh *et al.*, 1991a; Ruiz and Carrillo, 1993). Since *Glu-3*/*Gli-1* show almost complete linkage, the electrophoretic banding patterns of gliadin blocks coded at the *Gli-1* locus, extracted prior to the glutenins and run separately on SDS-PAGE, are indicative of the LMW-GS alleles present (Gupta and Shepherd, 1988; Singh *et al.*, 1991a; Gupta *et al.*, 1994). Molecular techniques have also been published that employ the polymerase chain reaction (PCR) to amplify gliadin DNA sequences. These dominant *Gli-1* PCR markers also provide an indication of which *Glu-3* allele is likely to be present (Zhang *et al.*, 2003). In addition, the more distant genetic linkage between *Glu-B3* alleles

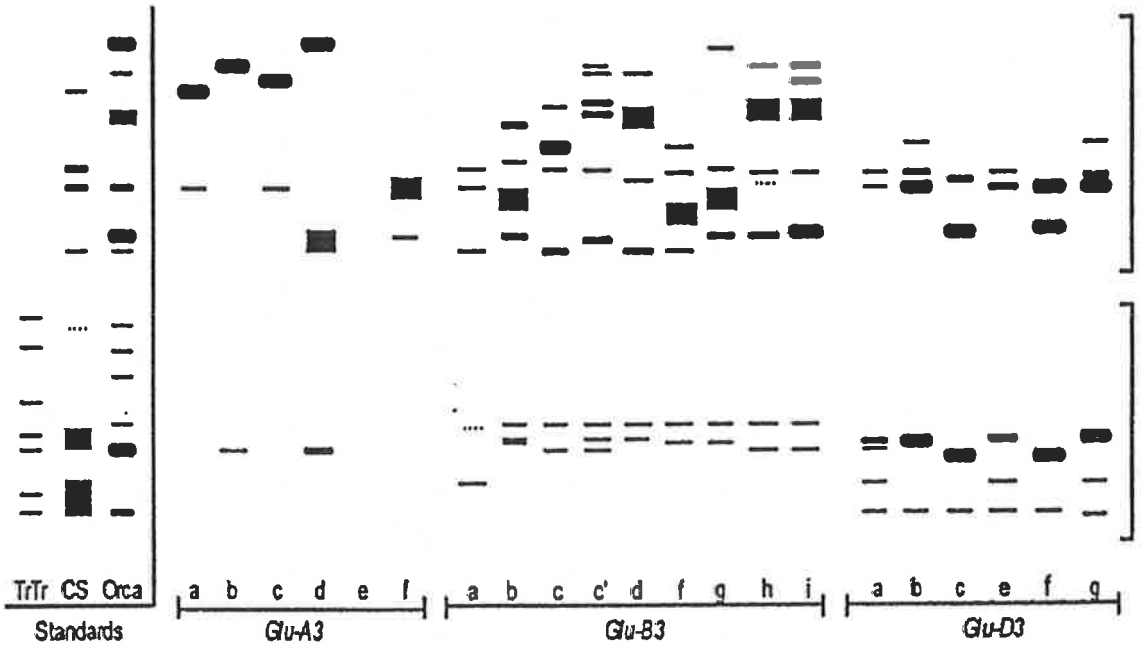


Figure 2-6 Simplified schematic representing the banding patterns of LMW-GSs and associated allelic designations (Gupta and Shepherd, 1990)

and the loci which determine glume colour, designated *Rg1* may also assist LMW-GS identification and selection. The *Rg1* dominant and recessive alleles determine brown (red/bronze) and white colours, respectively (Unrau, 1950). Positive correlations between glume colour, gluten strength and gliadin composition prompted investigations into the genetic linkage between *Gli-B1* and *Rg1* to ascertain if glume colour would permit selection for gluten strength in early generations (Payne *et al.*, 1986; Hare *et al.*, 1986). Payne *et al.* (1986) estimated the genetic distance between *Gli-B1* and *Rg1* to be 1.8 cM which equates to a genetic distance of 3.5 cM between *Glu-B3* and Rg1 (1.7 cM + 1.8 cM). Typically, cultivars containing LMW-GS alleles *Glu-B3c*, *Glu-B3g* or *Glu-B3i* have brown glumes while cultivars possessing other *Glu-B3* alleles have white/yellow glumes (Shepherd, pers. comm.).

In durum, three loci control the synthesis of LMW-GSs namely, *Glu-A3*, *Glu-B3* (Singh and Shepherd, 1988) and *Glu-B2* (Ruiz and Carrillo 1993; Liu 1995). Bread wheat LMW-

GS alleles have been classified differently to those in durum wheat which are broadly categorised into two main groups, directly related to quality and based only on the B-group LMW-GSs of slower electrophoretic mobility (Nieto-Taladriz *et al.*, 1997). The LMW-1 (LMW-1 and LMW-1$^-$) and LMW-2 (LMW-2, LMW-2$^-$ and LMW-2*) groups of proteins differ mainly by the presence of a subunit, associated with the LMW-2 group, with an 'apparent' molecular weight of 42 kDa (42 K subunit) on SDS-PAGE (Payne *et al.*, 1984b; Carrillo *et al.*, 1990). A-PAGE analysis of gliadins has shown that the LMW-1 group is linked to γ-gliadin 42 and to three ω-gliadin subunits 33, 35 and 38, whereas the LMW-2 group is linked to γ-gliadin 45 and ω-gliadin 35 (Masci *et al.*, 2000). Neither LMW-1 nor LMW-2 types have been detected in bread wheat cultivars, however a LMW-GS associated with the *Glu-B3h* allele in bread wheat shows a strong similarity to the 42 kDa subunit of the LMW-2 group (Masci *et al.*, 2000).

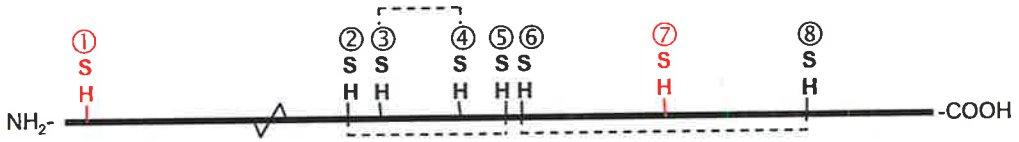
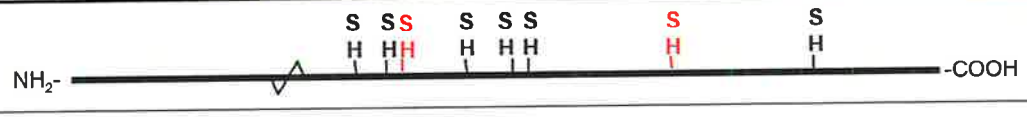
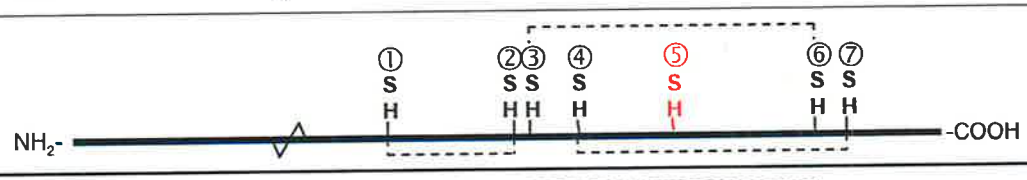
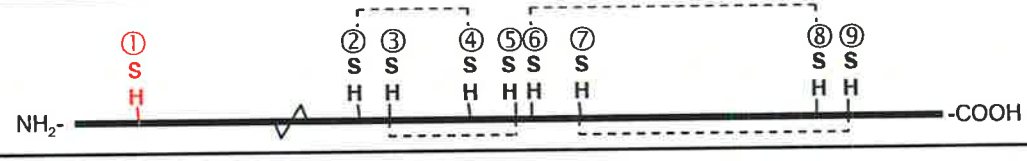
LMW-GSs have also been identified and classified according to amino acid sequence similarities rather than relative electrophoretic mobility on SDS-PAGE (Lew *et al.*, 1992). B- and C-group members of the complex LMW glutenin family fall into two broad classes. One class corresponds to those types with sequences similar to gliadins, (α- and γ-type). The other class comprises LMW-GSs where the first amino acid residue of the mature protein is either a methionine (LMW-m), serine (LMW-s) or isoleucine (LMW-i) (Cloutier *et al.*, 2001; Gianibelli *et al.*, 2001 Masci *et al.*, 2002; Ikeda *et al.*, 2002). Based on the alignment of deduced amino acid sequences from conserved N- and C-terminal domains, LMW-GS genes have been further classified into 12 groups (Ikeda *et al.*, 2002). Although in some LMW-m type LMW-GSs the first cysteine residue is at position 5 within the N-terminal conserved domain (groups 6, 7, 8, 9, and 10), in other LMW-m and LMW-s types, the first cysteine residue is found in the N-terminal repetitive domain (Lew *et al.*, 1992; Masci *et al.*, 1998; Ikeda *et al.*, 2002). The predicted N-terminal sequences of LMW-i type genes (groups 11 and 12) are truncated when compared to the LMW-m and LMW-s types.

As a result all of the eight cysteine residues are located in the C-terminal conserved domain (Cloutier *et al.*, 2001; Ikeda *et al.*, 2002). This is summarised in Table 2-2 showing N- and C-terminal sequences, the number and location of cysteine residues and putative cysteines involved in intramolecular disulfide bond formation. Investigations into the chromosomal location of genes encoding the different LMW-GS groups revealed that *Glu-A3* encodes groups 6, 11 and 12, *Glu-B3* encodes groups 2 and 3, and at *Glu-D3*, groups 1, 5, 7 and 10 are encoded (Ikeda *et al.*, 2002).

The elucidation of nucleotide sequences for LMW-GSs has led to significant advances in the development of PCR markers for specific LMW-GS alleles (Zhang *et al.*, 2003; Zhang *et al.*, 2004). These markers, especially when used in conjunction with SDS-PAGE, greatly enhance *Glu-3* identification and assist breeding programs to select for specific LMW-GS alleles.

2.3 Determination of Protein Quality Characteristics

Grain quality of cultivated wheat is determined by a complex matrix of variables, many of which are interdependent (Cracknell and Williams, 2001). Wheat has a wide range of end-uses and the required dough properties vary from one end product to another (MacRitchie, 1990). Hence, high grain quality can be defined as the optimum combination of functional properties for a given end product with the outcomes of quality testing providing an indication of a wheat's ability to produce it (MacRitchie, 1990). In Australia, quality testing of wheat varieties reflects the requirements outlined by the AWB for eligibility into one of its market grades. Table 2-3 summarises the sequence of events from genes to consumer, indicating opportunities for quality testing and provides some examples of the types of quality tests that are routinely conducted.

TYPE	GROUP	N-TERMINAL[a]	C-TERMINAL[a]	PRIMARY STRUCTURE[b]
LMW-m	1	METSHIPG	VGTQVGAY	LMW-mc5 (groups 6,7,8,9 and 10) 
	2	METSHIPS	VGTRVGAY	
	5	METSRVPG	IGTGVGGY	
	6	MDTSCIPG	VGTGVGAY	
	7	METSCISG	VGTGVGAY	
	8	METSCIPG	VGSRVGAY	
	9	METSCIPG	VGTQVGAY	
	10	METRCIPG	VGTGVGAY	
LMW-s	3	MENSHIPG	VGTGVGGY	
	4	IENSHIPG	VGAGVGAY	
LMW-i	11	ISQQQQQP	VGIGVGVY	
	12	ISQQQQQP	IGIGVGVY	
α–type		VRFPVPQL	PFGIFGTN	
γ–type		NMQVDPSS	VVAGIGGQ	

[a] The single-letter nomenclature for amino acids is as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

[b] Cysteine residues are marked SH. Putative cysteines involved in inter-molecular disulphide bonds are in red. Dashed line represents intra-molecular disulphide bonds.

Table 2-2 Comparison of the N-terminal amino acid sequence and primary structure of LMW-GS types (adapted from Lew *et al.*, 1992; Anderson and Greene 1997; Cloutier *et al.* 2001; Gianibelli *et al.*, 2001; Ikeda *et al.*, 2002)

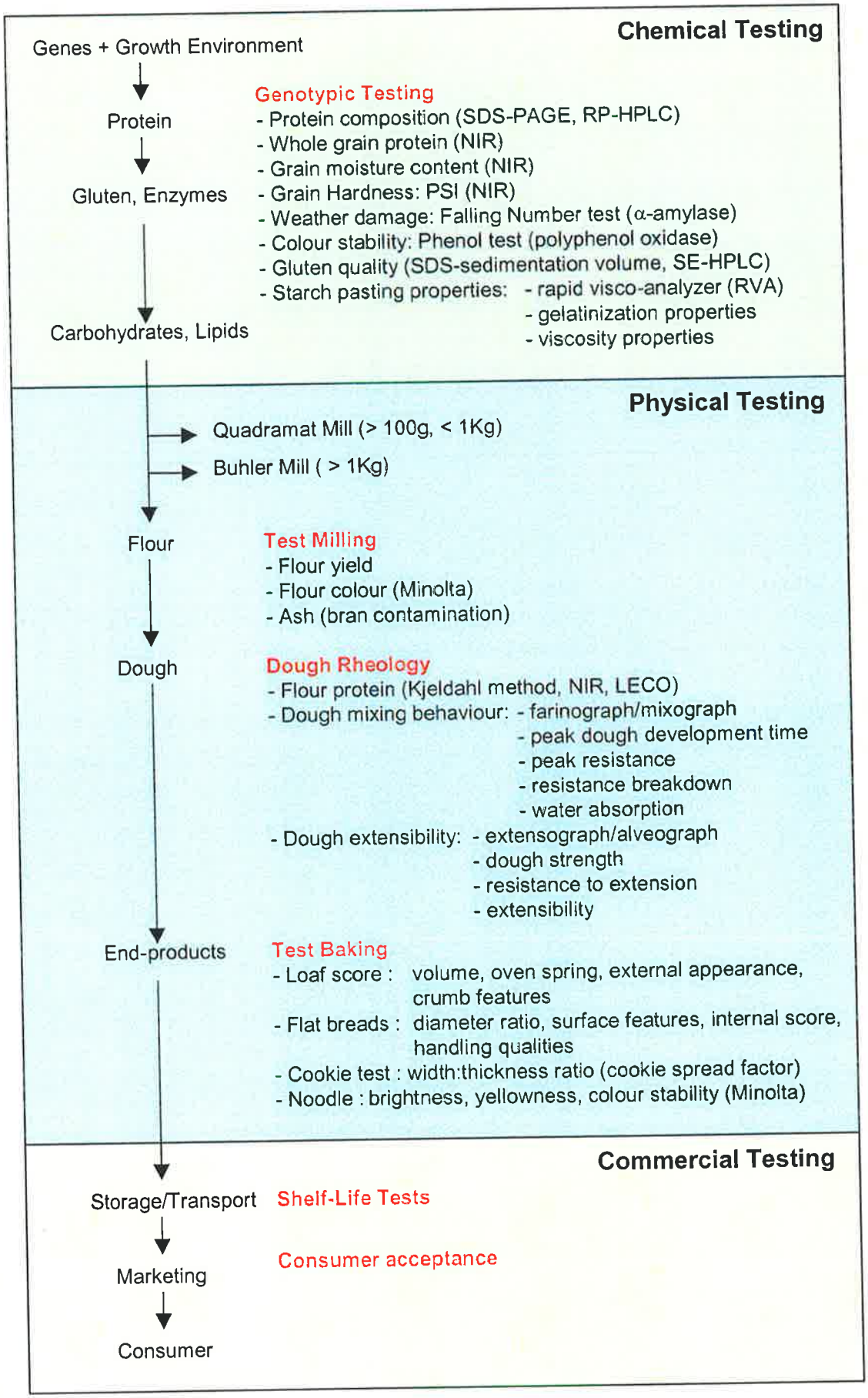


Table 2-3 Sequence of events from genes to consumer, indicating opportunities for quality testing and examples of some routine quality tests (adapted from MacRitchie, 1990)

2.3.1 Influence of dough rheology on bread making quality

For most traditional uses, wheat quality derives mainly from two inter-related characteristics; grain hardness and protein (amount and type), with each end-use requiring a particular 'quality' (Gianibelli *et al.*, 2001). Grain quality is typically measured by physical dough tests that determine its rheological properties. Dough rheology is related to functionality and rheological tests predict end product suitability, such as mixing behaviour, sheeting and baking performance (Dobraszczyk and Morgenstern, 2003).

When flour and water are mixed to form a dough there is an initial development stage where the coherent mass that is formed acquires visco-elastic properties. Recording dough mixers show a maximum resistance when the end of the development stage is reached. At this point the dough surface takes on a sheen and can be stretched into a thin sheet. This usually corresponds to the optimum mixing time for bread making and is referred to as peak dough development time. After this peak, further mixing causes a progressive decrease in the resistance, accompanied by the loss of elasticity and an increase in dough stickiness, both undesirable features for processing. This stage is referred to as dough breakdown (MacRitchie, 1992). Sheeting of dough with rolls after mixing is a common operation in the manufacture of cereal products. Bread dough is sheeted in a moulder to shape the dough and to control the bubble size distribution. Repeated sheeting can also be used to develop the gluten network and uses 10-15% of the energy required by mixing (Kilborn and Tipples, 1974; Dobraszczyk and Morgenstern, 2003). The greatest market demand is for varieties that have moderate to high dough strength and high extensibility, as these properties are best suited to the widest range of end products.

Doughs with these properties retain stability during mixing and hold the gas produced during fermentation as evenly distributed, discrete cells within their structure. The gas

cells expand during dough proofing and the early stages of baking, resulting in a loaf crumb structure where the gas cells are of regular size and even distribution. Overstrong, inextensible doughs are tough and rubbery. Their resistance to expansion during proofing and baking is great and the resulting loaves are of poor volume and texture. Weak doughs tend to breakdown during mixing. In these doughs the gas cells expand excessively during fermentation and proofing, causing their walls to collapse and the cells to coalesce. The resulting bread has a very open texture with a coarse wall structure (Simmonds, 1989). Flours from differing genotypes containing similar protein levels can vary in the time their doughs take to reach peak development and in steepness of the breakdown, a measure of mixing stability and rheological properties. In these cases, the variability is governed by differences in protein composition (MacRitchie, 1992).

2.3.2 Physical dough testing

Determination of dough rheological parameters can be achieved using various instruments, those most commonly employed include the farinograph, mixograph, extensograph and alveograph. The farinograph and the mixograph record, in graphical form, changes in the force required to mix a dough as it develops under standardised conditions. Both instruments consist of a pair of motor driven mixing blades which are mechanically coupled to a strip-chart recorder which records resistance to mixing as a function of time (versions of these machines that measure and record electronically also exist). The difference between farinographs and mixographs lies in their mixing action. A farinograph comprises Z-shaped blades that rotate in opposite directions so that dough is folded and squeezed between them. The pressure on these blades (ie. torque required to turn them) is measured to determine dough development time (FDDT), dough breakdown (FDBD) and water absorption (FWA). In a mixograph, the mixing head carries two pairs of pins that rotate between three pins fixed into the bottom of the mixing bowl. The mixograph also records dough mixing properties (Figure 2-7B) and measures dough development time or

time to reach peak resistance (MDDT), maximum peak resistance (MPR) and resistance breakdown (MRBD) (Simmonds, 1989).

Extensographs measure extensibility (Ext.), maximum resistance (R_{max}) and dough strength (Area) (Figure 2-7A). As a dough is stretched, protein network strands become thinner until a point is reached when they can no longer support the applied stress, and rupture occurs. Ext. corresponds to the elongation at the break in tensile strength (Figure 2-7 A). It should also be pointed out that Ext. depends on the state of development of the dough. In standard methods for measuring extensibility (AACC method 54-10, 1987), doughs are mixed for a fixed time to a fixed consistency. Since different flours have different mixing requirements for optimum development, this can mean that their doughs are not being compared at the same state of development. If a flour has very long dough mixing requirements, it may give a relatively low extensibility, because the optimum protein network has not had sufficient time to form (MacRitchie, 1992). This would also apply to standard test baking protocols. R_{max} is the maxiumum resistance or the force required to stretch the dough. While the area under the curve (Area) describes dough strength or the balance between Ext. and R_{max}, (Gupta *et al.*, 1989; Simmonds, 1989; Sapirstein and Fu, 1998).

The alveograph is not often used in Australia, but is widely used in other countries particularly France, so its inclusion here is simply to enable comparisons between investigations that report the effects of glutenin subunits on dough rheology. The alveograph measures the functional properties of a dough using parameters which include work input or gluten strength (W); tenacity or maximum resistance (P); swelling (G); and extensibility (L) (Figure 2-7 C). The strength is proportional to the area under the curve and the swelling is calculated from the extensibility. The P/G or P/L ratio (G and L are physically related) provides a better assessment of dough quality than these individual

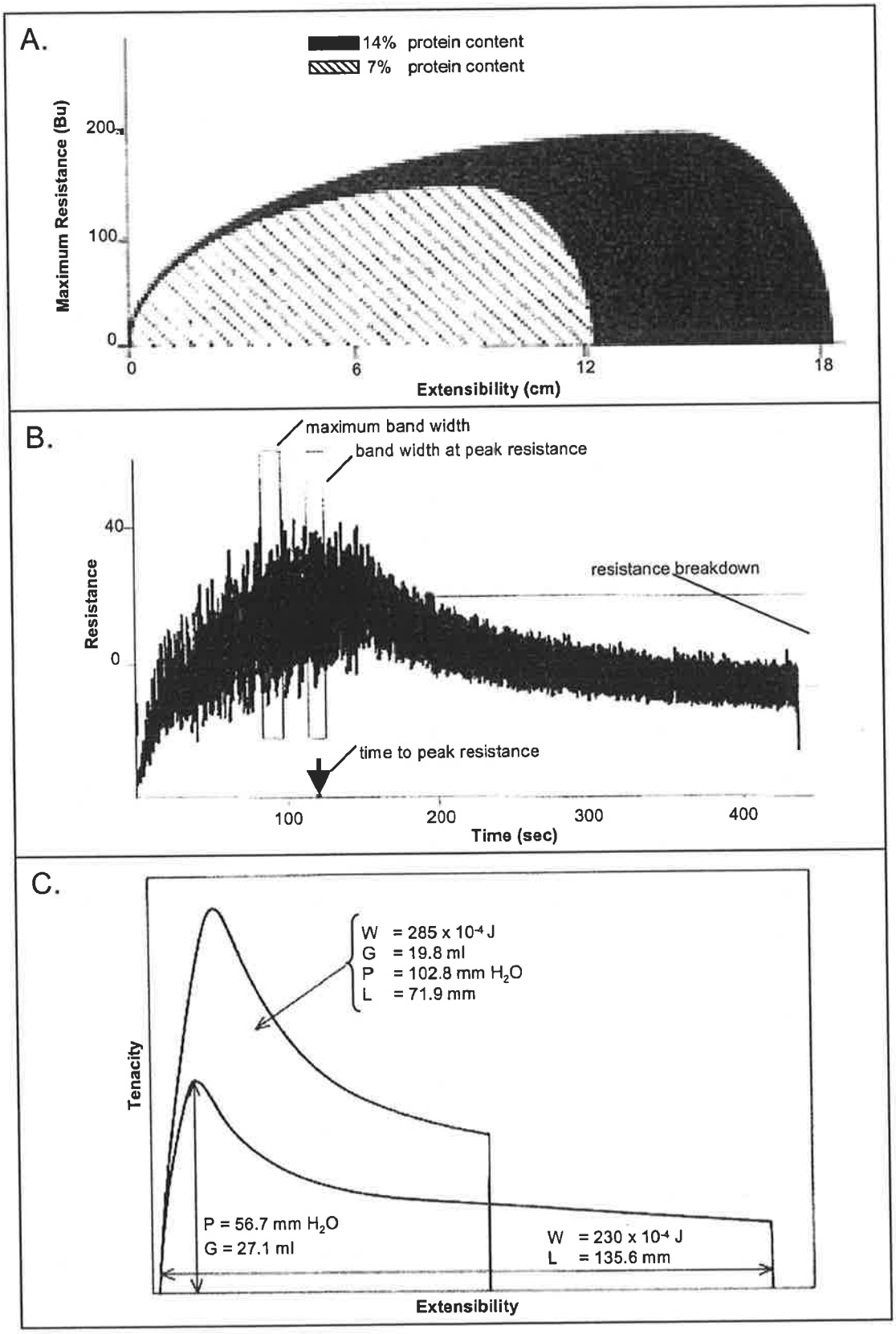


Figure 2-7 Data output from dough rheology testing. **A.** Extensograph measurements of maximum resistance (R_{max}) and extensibility (Ext.) (Gupta *et al.*, 1989). **B.** Mixograph variables. The x-axis tracks the time since the start of mixing and the y-axis measures the instantaneous levels of power required to mix the dough. The width of the trace reflects the repeated stretching and rupture of the dough as it is mixed (Gras *et al.*, 2001). **C.** Alveograph output for two cultivars of medium ($W=230 \times 10^{-4}$) and good ($W=285 \times 10^{-4}$) quality (Branlard and Dardevet 1985b).

parameters alone. Bread wheat that has strong, extensible gluten characteristics has a W value $\geq 350 \times 10^{-4}$ J and a low P/G (or P/L) ratio (Branlard and Dardevet, 1985a and b; Pena *et al.*, 1995).

Investigations into the statistical relationships among quality attributes revealed that FWA provides an indication of flour protein content (FP), grain hardness and the degree of damage to starch granules that occurred as a result of the milling process (Simmonds, 1989; Pomeranz, 1990; Singh *et al.*, 1990b). FP is also positively correlated with Ext. and FDDT but is negatively correlated with FDBD. Extensograph data (Ext., R_{max} and A) positively correlated with FDDT and MDDT and negatively with FDBD. A very strong correlation between R_{max} and MDDT reflects the fact that both measure dough resistance (Singh *et al.*, 1990b). A significant positive correlation was also found between loaf volume (LV), Ext., R_{max} and Area as well as MDDT (Singh *et al.*, 1990b). Importantly, R_{max} was found to be independent of FP. Also FP, milling yield (MY) and FWA were not significantly correlated with LV. The lack of correlation between FP and LV observed by Singh *et al.* (1990b) for different cultivars at similar FP indicated that differences in bread making quality were mainly due to difference in protein quality. This is in accordance with Finney and Barmore (1948) who observed that, although a strong linear relationship existed within a cultivar between FP and LV, large differences existed in the slopes of these relationships between cultivars (Figure 2-8). Clearly, genetic composition plays an important role in determining a wheat variety's ability to produce good quality breads.

2.3.3 Determination of polymeric protein

Recent use of size-exclusion high-performance-liquid chromatography (SE-HPLC), enabling protein separation based on size, to analyse wheat flour proteins has been successful (MacRitchie, 1999). In part, this is due to its requirement for small sample sizes so it can be used as an early generation screening tool. Other factors that contribute the

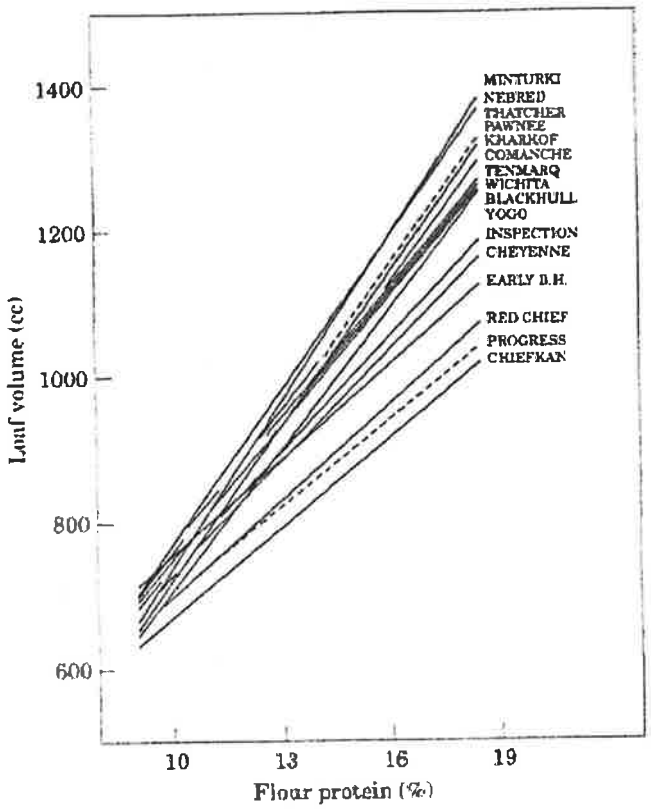


Figure 2-8 Variation in baking quality (as loaf volume) with protein content for flour samples of two wheat classes of different quality. —— Hard winter wheat and – – – Hard spring wheat (Finney and Barmore, 1948)

success of SE-HPLC are its reproducibility, automation and quantification capabilities (Singh *et al.*, 1990b; Bietz, 1992). Negative aspects associated with SE-HPLC include the requirement for expensive specialised equipment and high running costs.

SE-HPLC was first applied to wheat proteins by Bietz (1984). Analysis of unreduced SDS extracts from wheats differing in bread making quality revealed an inverse relationship between the amount of easily extracted HMW native glutenin and dough quality. Wheat with strong dough characteristics contains less acetic acid- or SDS-soluble glutenin and more SDS-insoluble glutenin in their flour (Singh *et al.*, 1990b). Hence, the amount of insoluble, often termed residue or gel protein, correlates positively with dough strength, and is the basis of residue protein and sedimentation tests (SDS and Zeleny) for bread

making quality (Orth and Bushuk, 1972; Moonen *et al.*, 1982; Branlard and Dardevet, 1985b; Singh *et al.*, 1990b).

One problem associated with SE-HPLC is the variable extractability of unreduced proteins. To overcome this researchers have developed a simple way to completely extract unreduced flour proteins using mechanical shear with an ultrasonic probe (Singh *et al.*, 1990a and b). In this system, the largest glutenin polymers break down into smaller polymers, facilitating their extraction, but they remain large enough to be separated on the basis of size from the monomeric gliadin and albumin/globulin fractions (Singh *et al.*, 1990b; MacRitchie, 1999). The percentage of protein extracted in this manner, termed SDS-unextractable polymeric protein (%UPP), analysed using SE-HPLC positively and significantly correlates with extensograph R_{max} (Figure 2-9) and bread- making quality of many diverse wheat genotypes (Singh *et al.*, 1990b; Gupta *et al.*, 1993; Wrigley, 1993; Ciaffi *et al.*, 1996a).

2.4 GROWTH ENVIRONMENT AND PROTEIN COMPOSITION

Knowledge of when different protein fractions are deposited during grain development has assisted in assessing the impact stresses such as nutrient, disease, high temperatures and drought on grain quality at maturity (Stone and Nicolas, 1996). Environmental variation associated with quality often exceeds genotypic variation by altering the rate and duration of protein deposition and also the protein composition of the mature wheat grain. (Lukow and McVetty, 1991; Peterson *et al.*, 1992; Ciaffi *et al.*, 1996b).

2.4.1 Water and nutrient availability

Many factors can produce environmental modifications in grain quality, including moisture and nutrient levels, particularly nitrogen and sulphur which effect protein content and the types of proteins stored, respectively (Paredez-Lopez *et al.*, 1985; Randall and Wrigley,

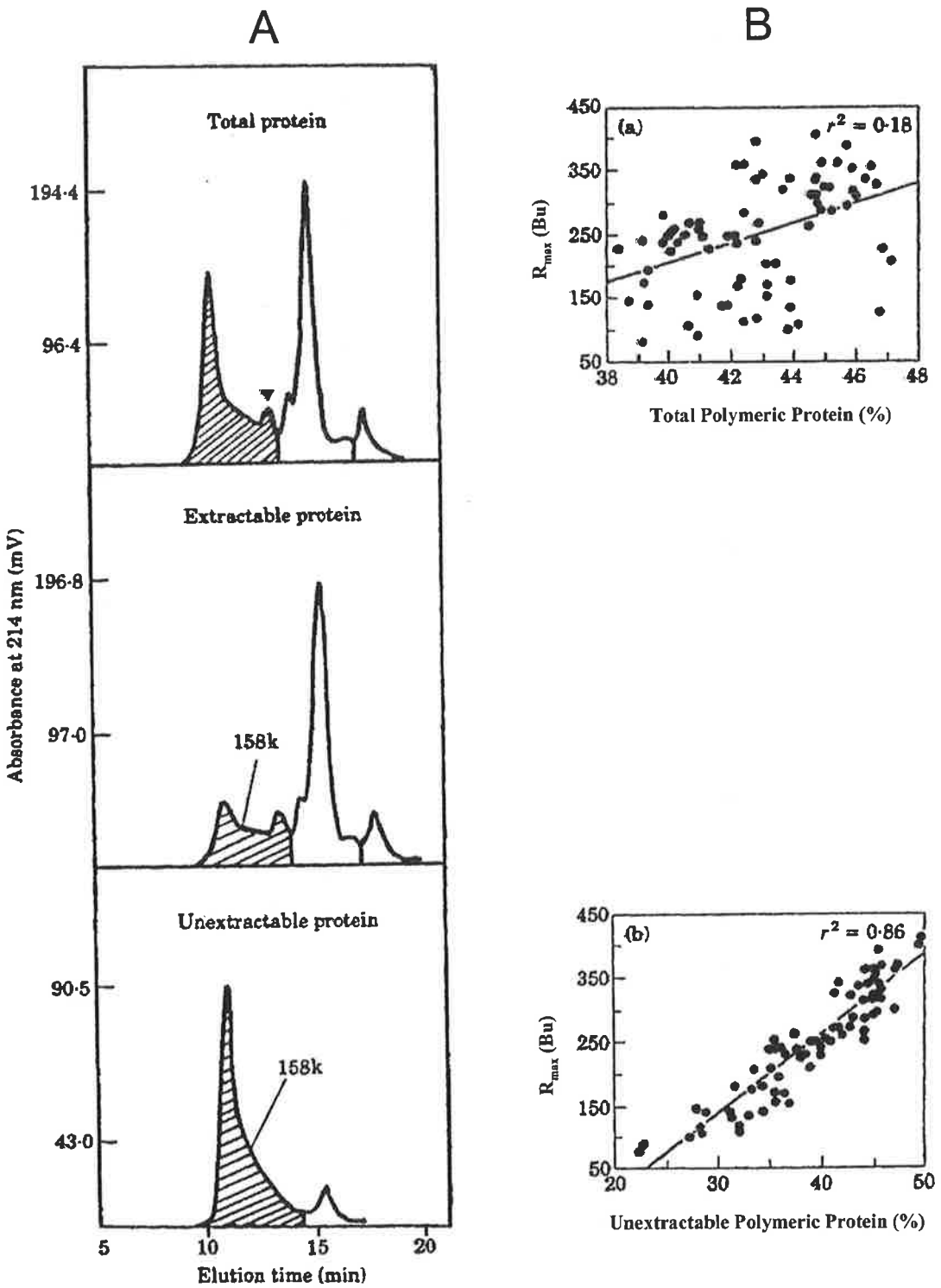


Figure 2-9 **A)** SE-HPLC separations of total, extractable and unextractable protein. Shaded area indicates polymeric protein. ▼ refers to ω-gliadin co-eluted with extractable polymers in the latter part of peak 1. **B)** Relationship between R_{max} and percentage of total (a) and unextractable (b) fractions of polymeric protein (Gupta *et al.*, 1993)

1990; MacRitchie and Gupta, 1993; Ciaffi *et al.*, 1996b). A high quantity of nitrogen available during grain filling increases protein deposition, while excessive rainfall throughout the growing season allows crops to produce greater yields resulting in a decrease in grain protein content due to a 'dilution effect'. Sulphur is required specifically for the synthesis of the amino acids cysteine/cystine and methionine. Under conditions of moderate to severe sulphur deficiency, the wheat plant modifies protein composition by synthesizing more of the low-sulphur group, particularly ω-gliadins, and less of the protein species containing higher amounts of sulphur (α-type gliadins, γ-type gliadins and LMW-GSs). Wheat samples, which contain more ω-gliadins, produce doughs that have an excessive resistance to extension and reduced loaf volume (Simmonds, 1989; Tatham *et al.*, 1990).

2.4.2 Temperature stress

The temperature during grain filling is probably the most important environmental factor affecting grain quality (Randall and Moss, 1990). As the temperature during grain filling increases the relative proportion of protein to starch increases. The optimum temperature range for reaching maximum kernel weight is 16-21°C; in this temperature range the absolute amount of protein per grain increases without a change in the amount of starch. Although, a temperature increase to 30°C reduces protein synthesis, it affects starch synthesis to a greater extent, resulting in an increase in grain protein content due to a suppression of starch synthesis (Kolderup, 1975; Sofield *et al.*, 1977; Hawker and Jenner, 1993; Ciaffi *et al.*, 1996b).

Temperature can also affect the functional properties of a dough without an accompanying change in protein content (Randall and Moss, 1990). At higher temperatures during grain filling, protein biosynthetic pathways in the grain are altered, leading to changes in protein composition (Blumenthal *et al.*, 1993). Randall and Moss (1990) examined cultivars

known to differ in their inherent dough properties and concluded that moderately high temperatures (25-32°C) during grain filling tended to improve the bread making quality of wheat via an increase in FP. In contrast, episodes of very high temperatures (>33°C) generally reduced the bread making quality of wheat despite an increase in protein content. These changes in grain quality were independent of flour protein and related to heat-induced changes in protein composition (Blumenthal *et al.*, 1991 and 1993; Wrigley *et al.*, 1994; Stone *et al.*, 1996; Ciaffi *et al.*, 1996b). Figure 2-10 shows the effect of mean cumulative hours above 35°C during grain filling on dough strength (R_{max}) for NSW crop samples of Prime Hard wheat for seasons 1960 to 1989 (Blumenthal *et al.*, 1991).

The relationship between heat stress and dough weakening can be caused by an increase in the ratio between SDS-soluble and SDS-insoluble protein polymers and/or an increase in the gliadin/glutenin ratio in (Blumenthal *et al.*, 1991; Ciaffi *et al.*, 1996b). The greatest accumulation of SDS-insoluble polymer coincides with the greatest decrease in SDS-

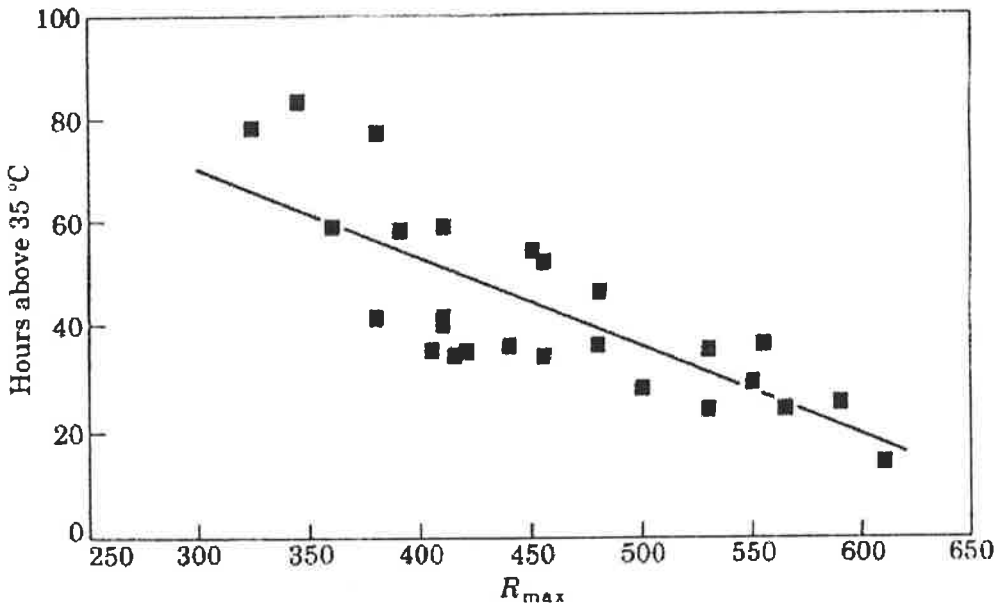


Figure 2-10 Variation in dough strength (R_{max}) for NSW crop samples of Prime Hard wheat for seasons 1960 to 1989 with mean cumulative hours above 35°C during 75 days starting 1 October at three sites (Moree, Myall Vale and Narrabri). The correlation co-efficient for this data was -0.787 ($P < 0.001$). Reproduced from Blumenthal *et al.* (1991 and 1993).

soluble polymer, suggesting that disulfide bonding of SDS-soluble polymer leads to the formation of SDS-insoluble polymer (Stone and Nicolas, 1996).

Changes in the composition of polymeric fractions due to higher temperature could result from the effect it has on post translational processes such as selective destabilisation of secretory protein mRNAs (including gluten proteins) or reduced efficiency of enzymes involved in the formation of intermolecular disulfide bonds (eg. protein disulfide isomerase) (Ciaffi *et al.*, 1996b). In addition, heat-shock elements (HSEs) upstream of the coding region of certain gliadin proteins have been identified. During a period of sudden heat stress, HSEs enable gliadin synthesis to continue at a greater rate than glutenin synthesis so that mature grains have a higher gliadin:glutenin ratio and produce weaker doughs (Blumenthal *et al.*, 1990a and 1990b). When there is a gradual increase in temperature, gluten proteins are produced at a constant rate and no change in the gliadin:glutenin ratio is evident at maturity (Ciaffi *et al.*, 1996b).

Reductions in dough strength resulting from heat stress during grain filling occur irrespective of the timing of the stress, but the magnitude of the decrease is greater if it occurs during the period of SDS-insoluble polymer formations. For the Australian wheat cultivar, Egret, this was 35-50 days after anthesis (Randall and Moss, 1990; Stone and Nicolas, 1996). The asynchronous nature of protein deposition and the existence of varietal differences in rate of polymer accumulation may explain why heat stress can have a greater effect on protein composition and dough strength in some wheat varieties rather than others (Blumenthal *et al.*, 1995; Stone and Nicolas, 1996). Also, the stability of dough quality to heat stress may be influenced by HMW-GS composition as the stability appears to be greater in wheat varieties that possess *Glu-D1d* (5+10). This can most likely be attributed to the extra cysteine residue in the 1Dx 5 subunit that is not present in other

subunits, allowing it to participate more effectively in the formation of polymeric protein (Shewry *et al.*, 1992).

2.5 GLUTENIN GENOTYPE AND BREAD MAKING QUALITY

Allelic variation exists at each of the loci that code gliadin and glutenin subunits and this has major implications in respect to protein quality and bread making potential. At least 20 major HMW-GSs have been identified by SDS-PAGE in surveys of cultivated and landraces of bread wheat. Similarly, over 70 alleles are known to exist for the three *Glu-3, Gli-1* and *Gli-2* loci (Colot, 1990).

2.5.1 HMW-GS composition

Improved methods of protein separation and resolution on SDS-PAGE combined with sedimentation tests, HPLC (size-exclusion and reversed-phase) and dough rheological testing has enabled researchers to study the protein composition of wheat cultivars. The ease with which HMW-GSs can be distinguished by SDS-PAGE has facilitated research into their effect on bread making quality. Pioneering work by Payne and co-workers established that HMW-GSs impart different effects on gluten quality. Most notable is the allelic variation at the *Glu-D1* locus and the contrasting effects of the alternative subunit pairs 5+10 and 2+12 (Payne *et al.*, 1979 and 1981). The results reported by Payne *et al.* (1979 and 1981) for British-grown varieties were later confirmed using other European wheats (Burnouf and Bouriquet, 1980; Moonen *et al.*, 1983; Branlard and Dardevet, 1985b) and some Australian cultivars (Lawrence *et al.*, 1987) have had a major impact in local breeding programs with the sustained release of varieties with stronger dough properties.

In addition to developing the numbering system used to identify HMW-GSs, Payne *et al.* (1987) devised a scoring system for HMW-GSs whereby alleles were graded according to

Score	Locus		
	Glu-A1	*Glu-B1*	*Glu-D1*
4	-	-	5+10
3	1	17+18	-
3	2*	7+8	-
3	-	13+16	-
2	-	7+9	2+12
2	null	-	3+12
1	-	7	4+12
1	-	6+8	-
1	-	20	-

Table 2-4 Quality scores assigned to individual HMW glutenin subunits or subunit pairs present in some British-grown wheats (Payne *et al.*, 1987).

their effect on SDS-sedimentation volume. This *Glu-1* quality score, which is the sum of the contributions of each of the three HMW-GS loci (Table 2-4) provides an indication of gluten characteristics for a given variety (Payne *et al.*, 1987; MacRitchie, 1992). The subunit pair 5+10 coded at *Glu-D1* was assigned a maximum value of 4. Subunits 17+18, 7+8 and 13+16 coded at *Glu-B1*, have also been associated with high dough strength, while the null allele coded at *Glu-A1* as well as subunits 2+12 (*Glu-D1*) and 7, 6+8 and 20 coded at *Glu-B1* all correlated with weaker dough properties (Payne *et al.*, 1987; Gupta *et al.*, 1989; MacRitchie, 1992; Shewry *et al.*, 1992).

The *Glu-1* scoring system has proved to be of great value in recent decades of wheat breeding, forming a basis for selecting parent lines and progeny with suitable dough properties or, alternatively, for eliminating known undesirable combinations of subunits early in a breeding programme (Shepherd, 1988; Wrigley, 2003). *Glu-B1u* (7+8), *Glu-B1al* (7+8*) and *Glu-B1ak* (7*+8*) were not assigned quality scores because of an inability to routinely differentiate them from *Glu-B1b* and as a consequence, the quality score given to *Glu-B1b* can be misleading since it fails to account for differences in quality

associated with these four alleles and in particular, with *Glu-B1al* which exerts a large positive influence on both R_{max} and Ext. (Gianibelli *et al.*, 2001).

Investigations into the molecular mechanisms by which certain subunits confer superior dough properties has revealed that qualitative effects can be related to differences in :

1) the number of subunits encoded, such as the negative effect of null alleles where no gene products are expressed (*Glu-A1*c) or where only one subunit is expressed (*Glu-B1a*);
2) the expression levels of subunits, a factor highlighted by the positive influence of *Glu-B1al* where subunit *Glu-B1* 7x is over-expressed; and
3) their structure which in turn affects their ability to form polymers with other HMW- and LMW-GSs.

An example of this can be seen by the influence of modified x-type subunits which usually contain four cysteine residues. Namely, the positive effect the extra cysteine (total of five) present in the *Glu-D1* 5x subunit has on quality in contrast to the negative influence of subunit *Glu-B1* 20x which has only two cysteine residues, the other two having been replaced by tyrosines (Tatham *et al.*, 1991; Lafiandra *et al.*, 1993a; Mackie *et al.*, 1996b; Buonocore *et al.*, 1996a; Lafiandra *et al.*, 2000b).

Inconsistencies reported in the literature are likely to be due to the complex interaction of factors that define wheat quality. These factors include LMW-GSs, other genes involved in the fundamental aspects grain quality, genetic x environment interactions and epistatic interactions between the *Glu-1* and *Glu-3* loci (Gianibelli *et al.*, 2001; Eagles *et al.*, 2002a).

Very little has been reported regarding the effect of rare or HMW-GS alleles derived from wild wheat progenitors. Alleles from *T. dicoccoides* accessions that express both Ax and Ay subunits have been shown to exert a positive influence (Ciaffi *et al.*, 1991, 1993 and 1995). In addition, *T. dicoccoides* contains a large reservoir of high grain protein content (GPC) genotypes (Gerechter-Amitai and Grama, 1977; Avivi, 1978; and Nevo *et al.*, 1986). These high GPC genes act to increase grain protein independently from grain yield. The positive influence of the high GPC genes from *T. dicoccoides* on gluten quality has been reported in two hexaploid wheat lines, namely a NZ breeding line, V761-9-4-2 (Grama *et al.*, 1984) and a USA cultivar, Glupro (Humphreys *et al.*, 1998; Mesfin *et al.*, 1999). For more detail regarding the high GPC genes present in *T. dicoccoides* refer Joppa and Cantrell (1990), Kovacs *et al.* (1998), Khan *et al.* (2000), Chee *et al.* (2001) and Olmos *et al.* (2003).

Comparison of the quality characteristics of the synthetic hexaploids, which had different *Glu-D1*^{t} alleles in a common durum background showed that those possessing the HMW-GS combinations of 5^t+12^t and 1.5^t+10^t had better overall quality characteristics than those with other subunits (Pena *et al.*, 1995). Another study which compared the subunits 2.1^t+10^t and 2.1^t+12^t in a common bread wheat background revealed that 2.1^t+10^t had a negative effect on quality while the subunit combination 2.1^t+12^t had a positive influence on quality similar to that of the 5+10 HMW-GS pair present in cultivated bread wheat (Pena *et al.*, 1996).

2.5.2 LMW-GS composition

Some varieties have better quality than would be expected on the basis of their HMW-GS composition, while the quality of others is unexpectedly low given their HMW-GS composition (Payne *et al.*, 1987). LMW-GSs are present in gluten at about three times the

amount of HMW-GSs but have received far less attention due to difficulties associated with their identification. It is known that LMW-GSs play a significant role in gluten structure and that the presence of certain alleles is positively correlated with good bread making potential and that the effects associated with these HMW-GSs and LMW-GSs are additive. (Payne *et al.*, 1987; Gupta *et al.*, 1989; Gianibelli *et al.*, 2001)

Inaccuracies in assessing the quality of Australian wheat varieties with a relatively high average *Glu-1* score of 8.6 results from variations in their *Glu-3* composition (MacRitchie *et al.*, 1990). In a set of 48 Australian wheat cultivars, LMW-GSs accounted for 42% of the variation in quality compared to 19% for the *Glu-1* score (Gupta *et al.*, 1991). Many Australian cultivars that receive a high *Glu-1* score, carry *Glu-3* alleles of low ranks eg. *Glu-A3e* and *Glu-B3c* in Insignia, Heron and Halberd (Table 2-5 and Table 2-6). Therefore, to explain more fully differences in bread making quality, the variation of *Glu-3* alleles in addition to *Glu-1* alleles needs to be considered (MacRitchie, 1992).

In a manner analogous to Payne and co-workers (1987), a preliminary approach to ranking LMW-GS alleles in order of quality was reported by Gupta *et al.* (1991). This work involved multiple regression analysis of *Glu-1*/*Glu-3* loci and used the extensograph parameters, R_{max} and Ext. to develop predictive formulas (Gupta *et al.*, 1991; MacRitchie, 1992). The addition of *Glu-3* loci to multiple regression models significantly enhanced prediction equations for R_{max} and Ext.. Since the amount of glutenin significantly affects dough rheological parameters and LMW-GSs are quantitatively the major group of glutenin subunits, contributing considerably more than do HMW-GSs to total glutenin, it is not surprising that the LMW-GS alleles with high ranks were associated with a greater number and/or intensity of bands in SDS-PAGE (Gupta and Shepherd, 1990; Gupta *et al.*, 1991).

Variety	Year	Glutenin Genotype	*Glu-1* score
Federation	1901	1; 20; 5+10 : *e; c; c*	8
Nabawa	1915	2*; 7*+9; 5+10 *: e; b; a*	9
Bencubbin	1929	2*; 7*+9; 2+12 *: e; b; a*	7
Gabo	1945	2*; 17+18; 2+12 *: b; b; b*	8
Insignia	1946	1; 20; 5+10 : *e; c; c*	8
Olympic	1956	1; 20; 5+10 : *c; b; a*	8
Heron	1958	1; 20; 5+10 : *e; c; c*	8
Gamenya	1960	2*; 17+18; 2+12 *: b; b; b*	8
Halberd	1969	1; 7*+9/20; 5+10 : *e; c; c*	8
Condor	1971	2*/null; 7*+8; 2+12 : *b/c; b; b*	8/7
Egret	1973	null; 7*+8; 5+10 : *c; b; b*	8
Banks	1979	2*; 7*+8; 2+12 : *b; b; c*	8
Hartog	1982	1; 17+18; 5+10 : *b; h; b*	10
Spear	1984	1; 7*+9; 5+10 : *c; h; c*	9
Janz	1989	1; 7*+8; 2+12 : *b; b; b*	8

Table 2-5 Glutenin genotype and *Glu-1* score of some important Australian wheat varieties (O'Brien *et al.*, 2001; AWB, 2005).

Gene	Frequency at each locus (%)	R_{max} (BU)	Extensibility (cm)
Glu-A3b	23.6	297 ± 19	16.8 ± 0.5
Glu-A3c	46.5	280 ± 19	16.5 ± 0.5
Glu-A3d	4.6	311 ± 20	16.8 ± 0.5
Glu-A3e	25.3	272 ± 19	15.6 ± 0.5
Glu-B3b	49.4	315 ± 18	16.8 ± 0.5
Glu-B3c	0.3	170 ± 56	17.3 ± 1.5
Glu-B3d	2.6	301 ± 20	16.2 ± 0.5
Glu-B3g	5.2	318 ± 19	16.7 ± 0.5
Glu-B3h	40.4	290 ± 18	17.0 ± 0.5
Glu-B3i	0.2	287 ± 44	17.4 ± 1.2
Glu-B3j	1.5	253 ± 25	13.0 ± 0.7
Glu-D3a	8.0	294 ± 20	16.3 ± 0.5
Glu-D3b	41.9	287 ± 19	16.4 ± 0.5
Glu-D3c	50.1	276 ± 19	16.3 ± 0.5

Table 2-6 Alleles detected at the *Glu-3* loci, frequencies and estimates of R_{max} and dough extensibility for each allele from a model without interactions (Eagles *et al.*, 2002a)

More recently, Eagles *et al.* (2002a) reported genotypic values for *Glu-1* and *Glu-3* for R_{max} and Ext. predicted from quality data obtained from southern Australian breeding programs, using statistical techniques appropriate for analysing large unbalanced data sets. The predictions for LMW-GS alleles, derived from a model without interactions, are presented in Table 2-6 (Eagles *et al.*, 2002a). Expanding on the information obtained for *Glu-1* and *Glu-3* alleles, and to assist breeding programs, these predictions have since been entered into a simulator based on QU-GENE (Podlich and Cooper, 1998) to predict progeny arrays from specific crosses (Eagles *et al.*, 2002a and b).

Predictions of Eagles *et al.* (2002a) are consistent with previous findings for the *Glu-A3* locus (Gupta and Shepherd, 1988; Gupta *et al.*, 1989 and 1994; Luo *et al.*, 2001) and rank, for both R_{max} and Ext., as follows : *Glu-A3d* > *b* > *c* > *e*. *Glu-A3e*, the null allele for which there is no protein product, produced a lower extensibility than the other alleles, with the difference between *Glu-A3b* and *Glu-A3d* being significant and approaching significance between *Glu-A3b* and *Glu-A3c*. *Glu-A3e* also produced a lower R_{max}, but the difference was only significant in the comparison with *Glu-A3d*. The frequency of *Glu-A3e* is relatively high in Australian germplasm (25.3%), but has declined in recent years because of selection against the detrimental effect this allele has on dough properties (Table 2-5). A positive contribution to dough strength has been reported for the *Glu-A3* allele, *Glu-A3g*. This allele was first identified in the "Canadian Extra Strong" wheat variety, Glenlea and does not appear to be present in Australian germplasm (Lukow and Townley-Smith, 1995; Cloutier *et al.*, 2001).

At the *Glu-B3* locus, the R_{max} of doughs produced from cultivars that contain *Glu-B3b* or *Glu-B3g* are significantly stronger than from cultivars which possess the *Glu-B3c* allele (Gupta *et al.*, 1991; Eagles *et al.*, 2002a). *Glu-B3j* has been associated with low R_{max} and

Ext. values, suggesting that it is an undesirable allele; fortunately it is not common in southern Australian germplasm (Eagles *et al.*, 2002a). *Glu-B3j* is the result of a rye translocation on chromosome 1BS which results in the wheat *Glu-B3* LMW-GSs being replaced with rye secalins. As a consequence doughs produced from bread wheat containing *Glu-B3j* are weak and sticky (Cornish, pers. comm.).

Numerous alleles exist at the *Glu-D3* locus (Gupta and Shepherd, 1990), however due to difficulties identifying them, Australian germplasm has typically been classified as either *Glu-D3a*, *Glu-D3b* or *Glu-D3c*. As a consequence, Australian studies investigating the dough rheological properties associated with *Glu-D3* alleles are likely to be misleading. Eagles *et al.* (2002a) found no significant differences between *Glu-D3* alleles for either Ext. or R_{max} although predictions indicated that for R_{max} *Glu-D3a* > *b* > *c* (Table 2-6). Other studies indicate that for R_{max}, *Glu-D3b* = *a* >> *c* (Gupta *et al.*, 1991) and *Glu-D3b* >> *c* = *a* (Metakovsky *et al.*, 1990).

In durum wheat, LMW-GSs encoded at the *Glu-A3*, *Glu-B3* and *Glu-B2* loci are considered the major determining factor of pasta quality (Payne *et al.*, 1984a; Pogna *et al.*, 1990; Ruiz and Carrillo 1995a and b; Vasquez *et al.*, 1996; Nieto-Taladriz *et al.*, 1997). Of the two major classes, LMW-1 and LMW-2 models, LMW-2 is associated with higher quality and, as is the case for high ranking bread-wheat LMW-GS alleles, quality differences between these two LMW-types can at least in part, be explained by quantitative differences between the LMW-1 and LMW-2 glutenin subunit fractions (14% and 27% of total protein, respectively) (Autran *et al.*, 1987; Gupta and Shepherd 1990; Gupta *et al.*, 1991; Ruiz and Carrillo 1995b; Nieto-Taladriz *et al.*, 1997). Amino-acid sequencing of the 42 kDa subunit redolent of LMW-2, revealed that it is a LMW-s type subunit where the first amino acid of the mature protein is a serine. LMW-s type LMW-GSs are predominant

in both durum and bread wheat cultivars and the association between a LMW-s type subunit and the LMW-2 is evidence that the presence of LMW-s type subunits can play an important role in grain quality (Masci *et al.*, 2000).

2.6 STRATEGIES TO IMPROVE BREAD MAKING POTENTIAL

The potential of a wheat variety for bread making depends on the amount of very large protein polymers present in the gluten complex because they facilitate retention of gas by the dough and enable it to retain shape and texture after baking (Simmonds, 1989). Questions arise as to whether the positive influence on quality of certain glutenin components is a result of :

1) structural characteristics (such as size, number of cysteine residues and other amino acid differences which alter peptide biochemistry eg. hydrophobicity);
2) epistatic interactions (genotypic and environmental);
3) the amount of glutenin protein relative to other proteins (such as the monomer:polymer ratio, gliadin:glutenin ratio, HMW-GS:LMW-GS ratio and B-group:C-group ratio); or
4) a combination of all three (Marchylo *et al.*, 1992; Ma *et al.*, 2004).

Polymer size, as indicated by %UPP is a measure of the molecular weight distribution (MWD) of protein polymers. It positively correlates with dough quality and is influenced by the number of glutenin subunits, the number of intermolecular disulfide bonds each glutenin subunit can form and perhaps, due to steric and chemical factors by the propensity of individual cysteines to form disulfide bonds (Masci *et al.*, 1996). As pan bread manufacture requires strong and extensible dough, the way forward in relation to improving dough quality would be to ensure a high MWD of protein, keeping in mind that a MWD shifted too high may negatively affect extensibility (MacRitchie, 1999).

2.6.1 Modification of molecular weight distribution

Dough properties are governed by the amount of large polymeric proteins present in flour, which upon the addition of water, aggregate to form the "gluten complex". The effect of many HMW-GSs and LMW-GSs on dough rheological parameters has, to a large extent, already been established. The challenge now faced is determining which combination of glutenin alleles best suits specific end-uses.

2.6.1.1 Cysteine residues

Many gluten models have been proposed and disulfide bonds are nearly always an important feature because they are responsible for cross-linking gluten protein components (Graveland *et al.*, 1985; Kasarda, 1989; Keck-Gassenmeier and Wieser, 1996). Polymer size is modulated by the incorporation of chain extender or chain terminator subunits. The ratio between chain extenders and chain terminators is a major factor determining MWD and wheat flour quality characteristics. Cultivars described as having good bread making quality would possess a large number of subunits that act as chain extenders and a minimal number of chain terminating subunits (Masci *et al.*, 1996; Lafiandra *et al.*, 2000a).

The presence of *Glu-D1d* (5+10), especially in southern growing regions of Australia, is desirable as it has a positive influence on bread making quality. This has been attributed to the presence of an extra cysteine residue in the 1Dx5 subunit. The usual complement of cysteine residues in x-type HMW-GSs is four, two of which form intermolecular disulfide bonds, whereas in the 1Dx5 subunit the additional cysteine means that there are three cysteine residues which can form disulfide linkages with other protein subunits (Shewry *et al.*, 1992). Substitution of 2+12 with a 5+10 facilitates more disulfide bonds between protein subunits and results in the formation of more and/or larger protein polymers. This leads to an increase in the MWD of protein polymers and an increase in dough strength. For this reason, subunit 1Dx5 is considered to be a chain extender.

LMW-GSs can also act as chain extenders. Typically LMW-GSs have six cysteines involved in intramolecular disulfide bonds and two cysteines free to form intermolecular disulfide bonds (Table 2-2). This explains their ability to form both mixed polymers with HMW-GSs and polymers strictly composed of LWM-GSs (Kasarda, 1989; Cassidy *et al.*, 1998). However, some subunits present in glutenin preparations do not facilitate the formation of glutenin protein polymers. These subunits, which are not encoded at the *Glu-3* loci, have been termed gliadin-like LMW-GSs (ω-, γ- and α-type LMW-GSs). The gliadin-like LMW-GSs are modified gliadins, most likely via a single mutation event, that have either acquired or are missing a cysteine residue. As a result, these peptides have only one cysteine residue available for intermolecular disulfide bond formation and act as chain terminators of growing glutenin polymer chains. Wheat varieties that contain a large number of chain terminating subunits have a low MWD of protein polymers and poor gluten quality (Huebner and Wall, 1976; Dachkevitch and Autran, 1989; Tao and Kasarda, 1989; Gupta *et al.*, 1993; Masci *et al.*, 1993 and 1999; D'Ovidio *et al.*, 1995; Anderson and Greene, 1997).

Not all mutation events that occur in the genes encoding storage proteins are detrimental. The characterisation of a clone containing the gene sequence of an ω-secalin from rye, a homologous gene for ω-gliadins with no cysteine residues available for intermolecular bonding, revealed that a single mutation event, like a frameshift mutation, can produce two cysteine codons, indicating that at least some gliadin-like LMW-GSs could act as polymer chain extenders (Clarke and Appels, 1999). In addition, changes in the position of cysteine residues may inhibit intramolecular bonds between the two cysteines normally involved. These two cysteines would then be able to form intermolecular disulfide bonds and serve to increase polymer size (Masci *et al.*, 2002).

2.6.1.2 HMW-GS to LMW-GS ratio

Good bread making quality cultivars have relatively higher concentrations of HMW-GSs than poorer ones (Kruger *et al.*, 1988; Kipp *et al.*, 1996; Larroque *et al.*, 1997). The most effective way to increase HMW-GS composition is to increase the number of different subunits encoded and/or alter the level of expression of individual subunits. In the first instance, wheat breeders can already choose to avoid the selection of alleles which encode a reduced number of subunits or no protein product at all (*Glu-B1a* and *Glu-A1c*, respectively).

The most promising approach for increasing the number of HMW-GSs lies with the *Glu-A1* locus. Unlike many wild wheat progenitors, the *Glu-A1* y-type subunit in cultivated polyploid wheat is almost always not expressed. In the literature reviewed here, there was only one paper reporting the presence of an expressed *Glu-A1* 21*y subunit in the Swedish breeding lines, W29323 and W3879, although it was speculated that this allele had been unintentionally introgressed during breeding processes from the wild wheat progenitors *T. urartu* or *T. dicoccoides* which are known to have genotypes that express both *Glu-A1* type subunits (Randhawa *et al.*, 1997; Margiotta *et al.*, 1996).

Quality testing of flour derived from these lines showed they had superior functional properties which was attributed to the presence of both *Glu-A1* x- and y-type subunits (Johansson *et al.*, 1996; Margiotta *et al.*, 1996; Lafiandra *et al.*, 1998). Other studies involving the introgression of *T. dicoccoides* x- and y-type *Glu-A1* subunits into *T. turgidum durum* have also reached similar conclusions (Ciaffi *et al.*, 1996a; Singh *et al.*, 1998). Another advantage of introducing *Glu-A1* y-type subunits into bread wheat, relates to extensibility. There is evidence that suggests that increasing y-type HMW-GSs increases Ext. (Kipp *et al.*, 1996). This is important, especially in varieties that contain

Glu-D1d as this allele can have significant detrimental effects on Ext. if the FP content is low or when it is combined with alleles that do not positively contribute to Ext., such as null alleles.

With regard to altering the level of HMW-GS expression, one way this could be achieved would be to select for glutenin alleles known to over-express certain HMW-GSs. A naturally occurring source of over-expression is *Glu-B1al* (Marchylo *et al.*, 1992). This allele has been identified in cultivars released around the world, including Australia and is associated with exceptional dough quality conveying both high dough strength and extensibility (Marchylo *et al.*, 1992; Lukow *et al.*, 1992; D'Ovidio *et al.*, 1997; and Eagles *et al.*, 2004).

The reason for over-expression is still unclear. A 43bp insertion in the matrix-attachment region upstream of the gene promoter and an 18bp insertion in the coding region of *Glu-B1* 7x have been identified, but neither of these insertions appear to be responsible (Lukow *et al.*, 1992; Butow *et al.*, 2004). The over-expression of subunit *Glu-B1* 7x and the presence of the *Glu-B1* subunit 8*y are considered diagnostic for the *Glu-B1al* allele and are used to distinguish it, using molecular PCR markers or RP-HPLC, from the other alleles with subunits that have similar electrophoretic mobility on SDS-PAGE (Sutton, 1991; Marchylo *et al.*, 1992; Gianibelli *et al.*, 2002; Butow *et al.*, 2003). There is the possibility that there are two *Glu-B1* 7x gene copies associated with the *Glu-B1al* allele. If both gene copies are functional this could account for *Glu-B1* 7x over-expression (D'Ovidio *et al.*, 1997; Lukow *et al.*, 2002).

To summarise, the MWD of polymeric protein could be increased by increasing the proportion of HMW-GSs relative to other gluten proteins. Theoretically, it is possible to produce hexaploid varieties that contain a total of seven HMW-GSs instead of the usual

complement of five. To achieve this, breeders would need to select for the *Glu-B1al* allele, assuming that there are two functional copies of *Glu-B1* 7x and introgress *Glu-A1* alleles from *T. dicoccoides* that encode both x- and y-type subunits. If such a variety was produced it would contain two, three and two HMW-GSs encoded at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci, respectively. This, together with high quality LMW-GSs and an appropriate background (eg *Pin* genes for grain hardness) should provide significant quality advantages over the cultivars currently grown.

2.6.2 Implications of improving wheat quality

The need of domestic flour millers to source grain from higher quality grades in order to maintain protein levels for bread manufacture has negatively impacted Australia's ability to compete in the international market. It is the opinion of local millers that flour derived from Australian Prime Hard (APH) and Australian Hard (AH) wheat varieties has too high a mixing requirement and dough strength for their purposes. In addition, Asian noodles, steamed breads and Arabic flat breads account for two-thirds of the end-uses of Australian wheat exports. A consequence of these two factors is that little emphasis has been placed on increasing dough strength in recent years (Zhen, 1993). With wheat production increasing in a number of regions including Eastern Europe, the Middle-East and the Indian subcontinent, countries such as India, Kazakhstan and Ukraine have recently emerged as significant grain exporters while other countries such as Iran have become more self-sufficient reducing, their imports (Quail *et al.*, 2004). It is essential that, for Australia to retain current markets and gain access to new ones, significant advances in wheat quality need to be achieved.

Already, due to a lack of suitable varieties, Australia is excluded from a major bread production system used in Asia. This region is considered to have the greatest growth potential for wheat products, increasing in parallel with the increase in disposable income

of the people of this region. A "sponge and dough" system used in Singapore, Thailand, Philippines and Japan produces a bread which has a high volume, a very fine crumb structure and a light texture with a characteristic flavour that is in high demand. Wheat varieties for the "sponge and dough" process are sourced exclusively from the USA (Dark Northern Spring) and Canada (Canada Western Red Spring). The flour derived from these grades are high in protein and produce strong stable doughs. Some Australian wheat varieties eg. Kukri and Chara have shown good potential for this product however the challenge ahead for Australia is to meet this standard consistently (Quail *et al.*, 2004).

This thesis describes work that investigates glutenin subunits with a view to improving wheat quality. The first section evaluates the HMW- and LMW-GS alleles already present in bread wheat using isogenic lines (5 backcrosses) of the Australian cultivar, Aroona, produced by Dr K.W. Shepherd of Adelaide University. The Aroona isolines consist of single, double and some triple HMW- and LMW-GS allele substitutions which enabled the contribution to dough rheological parameters of individual glutenin alleles to be determined in a common background. Many HMW- and LMW-GSs present in this allele set are not commonly found in Australian germplasm so information regarding them would be useful to breeders. Whilst in the field (2000-2003), a nitrogen treatment was applied so that the grain produced by each isoline had two substantially different levels of FP. The purpose of this was to establish which glutenin alleles at low FP performed at least as well as others at a higher FP level.

The HMW-GS allele, *Glu-B1al* was not present in the Aroona isoline set as it was unrecognised at the outset of the backcrossing program, but the positive influence of this allele is well documented (Sutton, 1991; Marchylo *et al.*, 1992; Gianibelli *et al.*, 2002; Butow *et al.*, 2002). A RP-HPLC assay, based on previously published methods, was used to study elution and expression profiles of *Glu-B1al* and other HMW-GS alleles. The

expression levels of HMW-GSs, how this may relate to dough quality as well as the origin of the *Glu-B1al* allele is investigated.

Another aspect of work covered here, involves the screening of wild wheat progenitors for new glutenin alleles which may, when introgressed, enhance the dough quality of cultivated bread wheat. SE-HPLC and SDS-PAGE was used to screen synthetic hexaploid and *T. tauschii* accessions. While for *T. dicoccoides* accessions, a simple Turbidity assay was developed and used as a screening tool to identify accessions with favourable protein quality attributes. The Turbidity assay was based on the same principle as SE-HPLC but measures the percentage of total SDS-insoluble protein (%IP) as, it is reasonable to assume that for there to be a high %UPP there must also be a large %IP. SDS-PAGE was used to investigate the genetic variation associated with the *Glu-1* loci and to characterise new HMW-GS alleles in *T. dicoccoides* accessions. These experiments made it possible to select promising germplasm, from accessions of wild wheat progenitors and synthetic hexaploids, which possess new HMW-GS genes that have the potential to improve the bread making quality of Australian bread wheat.

CHAPTER 3

GENERAL MATERIALS AND METHODS

This chapter describes materials and protocols that were based on previously published methods and applied in the course of the work reported in this thesis.

3.1 GRAIN SAMPLES

3.1.1 Aroona isolines

Fifty-five Aroona recombinant inbred lines (Aril) were developed by Dr K.W.S. Shepherd, Department of Plant Science, The University of Adelaide, South Australia. The six glutenin alleles present in the recurrent parent, Aroona are *Glu-A1a* (1), *Glu-B1c* (7*+9), *Glu-D1a* (2+12), *Glu-A3c*, *Glu-B3b* and *Glu-D3c* and dough produced from its flour is considered to be of medium quality. This allowed for both the positive and negative effects that resulted from *Glu-1* and *Glu-3* allele substitutions to be readily observed. The isolines, derived by 5 backcrosses into Aroona, contained 11 HMW-GS and 19 LMW-GS single allele substitutions from donor varieties which were selected because they contained the particular glutenin genes of interest (Table 3-1). In addition to the single allele substitutions, combinations of double allele (22 DA lines) and triple allele (2 TA lines) substitutions (listed in Appendix II) were produced to identify and measure the extent of interactions between the various glutenin alleles. The isolines also contain substituted gliadin genes (*Gli-1*) and genes that determine glume colour (*Rg1*) which are located in close proximity to the *Glu-3* loci and are co-inherited (Hare *et al.*, 1986; Payne *et al.*, 1986).

The isolines were planted for four consecutive years (2000-2003) in field trials at Charlick Experimental Station, Strathalbyn, South Australia (Latitude: -35.29; Longitude: 138.89). The field layout was a split plot design, with isolines as whole plots and nitrogen treatment as sub-plots. Two replications were used in 2000 and 2001 and three in 2002 and 2003. In

each year after anthesis, foliar application of urea, equating to 5g/m^2 (25kg/ha) of nitrogen (N), was sprayed on to one of these sub-plots at weekly intervals. This resulted in two treatment groups (N nil and N spray), however due to variable seasonal factors, the final amount of nitrogen applied in each year was different (10g/m^2, 25g/m^2, 20g/m^2 and 15g/m^2 in 2000, 2001, 2002 and 2003, respectively). Average rainfall during the April to October period for each year was 398mL in 2000, 322mL in 2001, 253mL in 2002 and 453mL in 2003.

Isoline ID[a]	*Glu-A1*	*Glu-B1*	*Glu-D1*	*Glu-A3*	*Glu-B3*	*Glu-D3*	Donor Parent
Aroona	*a*	*c*	*a*	*c*	*b*	*c*	Aroona
Aril 2-4 (2*)	*b*	*c*	*a*	*c*	*b*	*c*	Sonalika
Aril 3-2 (null)	*c*	*c*	*a*	*c*	*b*	*c*	Chinese Spring
Aril 4-3 (3*)	*p*	*c*	*a*	*c*	*b*	*c*	BT2288A
Aril 5-2 (7*)	*a*	*a*	*a*	*c*	*b*	*c*	Orca
Aril 6-4 (7+8)	*a*	*b*	*a*	*c*	*b*	*c*	Chinese Spring
Aril 7-1 (7+8)	*a*	*b*	*a*	*c*	*b*	*c*	India 115
Aril 9-3 (6+8*)	*a*	*d*	*a*	*c*	*b*	*c*	Rendezvous
Aril 10-1 (17+18)	*a*	*i*	*a*	*c*	*b*	*c*	Gabo
Aril 12-3 (3+12)	*a*	*c*	*b*	*c*	*b*	*c*	Rendezvous
Aril 13-3 (5+10)	*a*	*c*	*d*	*c*	*b*	*c*	Halberd
Aril 14-3 (2.2+12)	*a*	*c*	*f*	*c*	*b*	*c*	Norin 61
Aril 15-4	*a*	*c*	*a*	*a*	*b*	*c*	Chinese Spring
Aril 16-1	*a*	*c*	*a*	*b*	*b*	*c*	Gabo
Aril 18-5	*a*	*c*	*a*	*d*	*b*	*c*	Orca
Aril 18-9	*a*	*c*	*a*	*d*	*b*	*c*	Lerma Rojo
Aril 19-2	*a*	*c*	*a*	*e*	*b*	*c*	Bungulla
Aril 20-1	*a*	*c*	*a*	*f*	*b*	*c*	BT2288A
Aril 21-2	*a*	*c*	*a*	*c*	*a*	*c*	Chinese Spring
Aril 23-4	*a*	*c*	*a*	*c*	*c*	*c*	Halberd
Aril 24-3	*a*	*c*	*a*	*c*	*d*	*c*	Orca
Aril 26-1	*a*	*c*	*a*	*c*	*f*	*c*	Gawain
Aril 27-6	*a*	*c*	*a*	*c*	*g*	*c*	Millewa
Aril 28-4	*a*	*c*	*a*	*c*	*h*	*c*	Sonalika
Aril 29-4	*a*	*c*	*a*	*c*	*i*	*c*	Jufy 1
Aril 27-3	*a*	*c*	*a*	*c*	*m*	*c*	Bungulla
Aril 30-1	*a*	*c*	*a*	*c*	*b*	*a*	Chinese Spring
Aril 34-1	*a*	*c*	*a*	*c*	*b*	*a-Gli-D1*$^{-}$	BT2288A
Aril 31-2	*a*	*c*	*a*	*c*	*b*	*b*	Bungulla
Aril 33-1	*a*	*c*	*a*	*c*	*b*	*d*	Jufy 1
Aril 35-1	*a*	*c*	*a*	*c*	*b*	*f*	India 115

[a] Aril denotes Aroona recombinant inbred line (5 backcrosses)

Table 3-1 Donor parents for the single allele substitutions in the Aroona isolines (Shepherd, pers. comm.).

3.1.2 Samples used to investigate HMW-GS expression levels

Seed samples for most of the wheat varieties used in this study were obtained from the Australian Winter Cereals Collection (AWCC), Tamworth, New South Wales. Seed of the varieties Laura, Biggar, Wildcat, Bluesky and Oslo was obtained from Dr OD Lukow, Agriculture and Agri-Food Canada, Cereal Research Centre, Winnipeg, Canada. Seed of the two New Zealand varieties, Kohika and Endeavour was supplied by Dr WB Griffin, New Zealand Institute for Crop and Food Research Ltd, Christchurch, New Zealand. The varieties (Table 5-3, Chapter 5) investigated here were subjected to RP-HPLC to determine HMW-GS expressions levels.

3.1.3 Samples used to develop and validate the Turbidity assay

Harvested grain from 52 bread wheat lines was provided by Australian Grain Technologies (AGT, Adelaide, South Australia). The lines were part of their 2001 calibration set grown at the University of Adelaide's Roseworthy Campus, South Australia (Longitude: 138.69E; Latitude :-34.53S) and sub-sampled so that a wide range of extensograph maximum resistance (R_{max}) was represented. Both hard and soft endosperm textures were present and the samples had been analysed by AGT for the following dough rheological parameters: Flour protein content (FP), extensograph R_{max}, extensibility (Ext.) and dough strength (Area), farinograph water absorption (FWA), dough development time (FDDT), dough stability (FDS) and dough breakdown (FDBD).

Parameters determined in this study included SE-HPLC total protein (TP), the percentage of total SDS-unextractable protein (%UP), the percentage of total polymeric protein in protein (%PPP), the percentage of total polymeric protein in flour (%FPP) and the percentage of SDS-unextractable polymeric protein in total polymeric protein (%UPP) as well as the percentage of SDS-insoluble protein in total protein (%IP) determined using the Turbidity assay. All quality, SE-HPLC and Turbidity assay results (Table 3-2) were

VARIETY	NIR		Farinograph				Extensograph			SE-HPLC					Turb.
	FP	PSI	FWA	FDDT	FDS	FDBD	Ext.	R_{max}	Area	TP	UP	PPP	FPP	UPP	IP
	(%)		(mL)	(min)	(min)	(min)	(cm)	(BU)	(cm^2)	(area)	(%)	(%)	(%)	(%)	(%)
CO4570-516	8.8	16	57.3	6.6	10.0	35.0	19.9	540	183.0	212	40.5	34.8	3.06	58.3	45.5
KUKRI	9.1	16	58.9	6.1	10.0	30.0	20.5	470	184.3	201	37.6	34.2	3.11	54.8	48.1
CO3232-703	8.3	21	57.9	2.5	9.1	20.0	17.4	430	156.5	196	35.6	35.7	2.96	53.1	39.3
CO3035-702	7.9	15	59.8	2.2	7.2	30.0	15.1	400	118.5	175	35.9	34.4	2.72	52.4	37.9
CO3960-217	10.3	15	59.1	4.7	8.2	35.0	20.6	395	159.8	228	39.0	34.8	3.59	54.8	46.1
WI95112	8.3	19	57.9	4.6	8.0	35.0	18.5	385	143.5	199	36.6	33.5	2.78	54.1	45.1
CO3718-202	9.2	16	58.2	4.0	7.0	40.0	19.0	370	145.3	209	35.9	34.4	3.16	52.1	45.2
VI184	8.4	15	58.4	2.7	7.4	30.0	17.0	350	113.0	194	38.1	34.3	2.88	54.2	42.4
V1341	7.4	22	55.5	2.1	5.4	40.0	18.7	345	130.8	173	36.7	34.6	2.56	54.0	41.7
906R-12-039	9.0	13	61.8	4.2	6.8	35.0	18.5	330	116.0	218	33.8	33.7	3.03	50.4	36.7
RAC 737	8.5	18	60.3	2.2	5.6	40.0	14.3	325	96.0	201	31.8	33.9	2.88	47.0	33.4
487W100	8.8	17	60.5	4.3	6.2	40.0	18.5	320	122.0	212	36.8	33.6	2.96	52.3	47.1
CO4417-440	8.1	12	60.8	4.0	6.3	40.0	15.2	315	91.8	197	34.7	33.5	2.71	50.7	42.2
CO4417-414	8.5	13	61.8	4.0	6.1	45.0	16.0	310	98.3	210	31.8	32.8	2.79	48.3	41.8
MACHETE	8.8	17	60.9	3.4	5.4	45.0	18.4	300	110.0	207	35.1	33.8	2.97	51.1	44.3
486W124	8.3	20	55.9	3.8	5.7	50.0	19.0	295	111.3	196	32.9	32.4	2.69	48.1	41.7
DT53814	7.9	22	56.5	2.5	4.0	75.0	16.7	285	92.0	187	35.1	33.9	2.68	51.0	36.8
EXCALIBUR	7.5	20	56.5	3.1	4.7	65.0	15.3	280	87.3	168	30.6	32.6	2.45	44.5	30.4
CO2928-606	7.9	16	57.4	2.4	4.2	50.0	13.9	270	76.0	191	30.7	32.3	2.55	45.0	33.9
MOLINEUX	8.8	15	60.7	3.5	4.3	55.0	20.0	265	104.6	207	31.4	34.6	3.05	47.3	31.9
91INTRO2-VF519	9.0	11	60.5	3.2	4.3	60.0	18.0	260	91.5	220	33.5	33.1	2.98	48.8	38.2
	8.8	18	60.4	3.5	5.2	35.0	20.5	255	106.8	202	32.7	34.2	3.01	48.4	37.9
CO4443-508	8.7	17	56.8	2.9	4.0	60.0	15.3	250	75.8	206	34.8	33.0	2.87	48.8	35.6
JANZ	8.0	18	58.4	3.1	4.8	50.0	16.2	245	85.3	195	33.7	33.4	2.67	49.1	44.3
CO3186-701	9.0	10	63.2	3.4	4.1	45.0	15.6	240	78.0	229	32.5	33.4	3.00	48.1	40.0
FRAME	7.5	16	59.4	2.2	5.2	45.0	14.0	230	69.0	190	29.3	32.1	2.40	45.2	37.2
STILETTO	8.5	15	60.8	3.4	4.4	55.0	16.1	225	72.0	190	30.1	34.1	2.89	45.8	34.3
RAC 725	8.6	12	62.8	3.1	3.5	60.0	15.0	220	68.0	201	32.6	33.7	2.89	47.6	36.6
RAC 711	9.1	14	58.7	3.2	4.6	50.0	16.5	215	73.5	207	32.4	33.1	3.01	47.9	35.5
TRIDENT	8.9	14	62.0	3.5	4.8	50.0	15.9	210	71.3	197	32.5	32.9	2.93	48.0	35.2
DD007	8.8	21	57.6	2.9	3.6	75.0	17.8	205	73.0	212	32.8	33.6	2.95	46.7	36.8
CO4057-123	9.1	15	59.8	3.8	4.1	55.0	15.1	200	66.3	207	28.3	28.2	2.57	47.4	31.6
RAC 719	8.0	13	59.6	3.0	3.6	60.0	15.6	195	64.3	190	30.8	32.9	2.63	44.6	28.6
CO4102-312	9.0	11	61.6	3.5	3.2	60.0	17.5	190	71.0	214	32.3	33.4	3.00	45.5	35.9
CO4520-506	8.5	11	62.8	3.2	3.5	50.0	12.3	180	47.0	200	31.6	27.7	2.35	48.8	32.6
CO3242-702	8.8	12	62.7	2.8	2.4	70.0	15.2	175	56.5	219	31.4	32.7	2.87	44.6	33.4
CO3257-703	8.7	19	60.4	2.5	4.2	60.0	16.1	170	60.0	197	28.9	33.1	2.88	42.3	31.5
MEERING	8.2	18	58.1	2.6	3.1	70.0	16.7	165	57.8	187	27.6	33.4	2.74	41.3	28.2
CO4136-302	7.7	13	61.3	2.8	2.7	65.0	15.6	160	53.5	184	29.1	33.6	2.59	40.7	31.4
WW2450	8.1	20	57.4	2.4	2.8	70.0	16.5	155	53.8	187	28.6	32.7	2.65	41.9	29.9
RAC 712	8.3	21	56.0	2.4	2.6	75.0	15.5	150	50.8	196	26.2	32.5	2.70	39.3	27.4
906R-11-072	7.4	15	58.1	2.3	2.3	75.0	14.6	145	46.5	188	30.8	34.5	2.55	44.4	35.1
RAC 731	7.8	18	61.6	2.2	2.6	90.0	13.7	140	43.5	178	28.0	33.3	2.59	41.1	29.3
CO3258-756	8.4	25	54.7	2.3	2.0	100.0	17.3	135	52.0	205	25.7	32.1	2.69	38.8	19.0
LILLIMUR	8.0	24	57.9	2.4	2.3	95.0	16.2	130	45.0	184	26.5	34.1	2.73	38.6	25.3
CO-4443-512	8.2	26	53.9	2.0	1.7	115.0	15.8	125	44.3	179	24.3	31.6	2.59	35.2	23.3
WW2449	7.2	22	56.3	2.1	1.6	95.0	15.4	120	41.8	172	27.2	32.1	2.31	40.5	31.0
RAC 735	8.1	22	56.6	2.4	2.0	90.0	16.5	115	43.0	182	23.0	33.5	2.71	34.3	23.6
CO3273-711	8.0	19	59.9	1.9	1.7	100.0	14.8	110	32.0	193	24.3	32.2	2.58	35.0	22.0
CO3590-244	8.8	18	58.2	2.1	2.4	70.0	19.1	105	44.5	204	32.0	33.9	2.98	42.8	33.1
RAC 721	7.6	13	62.0	2.2	2.0	95.0	15.1	95	33.8	183	27.3	31.9	2.42	39.2	24.6
TINCURRIN	7.6	31	53.1	1.8	1.4	135.0	15.1	90	25.3	169	19.5	29.2	2.22	30.5	13.5

Table 3-2 Results of NIR, Farinograph, Extensograph, SE-HPLC and Turbidimetric analysis conducted on Buhler milled flour derived from 52 hexaploid varieties grown at the University of Adelaide, Roseworthy Campus, SA in 2001.

analysed using standard statistical methods. Determination of means, linear regressions, correlation coefficients and analysis of variance was performed using Microsoft Office Excel 2003. SE-HPLC and Turbidity assays were conducted in duplicate and the means reported.

3.1.4 Novel glutenin alleles in wild wheat progenitors

Accessions of *T. tauschii* and synthetic hexaploids had been obtained from the AWCC prior to the commencement of this work, making them readily available to study here. The 66 *T. tauschii* accessions were originally part of a collection held by the Australian Commonwealth Scientific and Research Organisation (CSIRO, Canberra, Australian Capital Territory). With the exception of AUS 24130, from Afghanistan, they all originated from Iran and both *T. tauschii* ssp. *eusquarossa* (var. *typica* and *meyeri*) and ssp. *strangulata* (var. *strangulata*) were present.

The 148 synthetic hexaploid (*T. durum* x *T. tauschii*) accessions obtained from the AWCC were originally produced at the International Maize and Wheat Improvement Centre (CIMMYT). During the late 1980's, wide crosses research at CIMMYT was expanded to include inter-specific hybridisation to exploit the genetic variability residing in the three genome donors of modern cultivated hexaploid wheat. As a result, more than 500 synthetic wheats with A-, B- and/or D-genomes derived from sources other than bread wheat have been produced (Skovmand *et al.*, 2002). Of the 148 accessions investigated here, 61 were primary synthetic hexaploid lines, while the remaining 87 were derived bread wheat lines containing at least one synthetic hexaploid in their pedigree.

T. dicoccoides accessions were requested from the AWCC during the course of this project. In response, 335 accessions were received in 2001. 271 of these (81%) were part of Kushnir's collection with 268 of them originating from Israel while the other 3 came

from France. The remaining 19% (64 out of 335 lines) were sourced from various collections and comprised 33 accessions that originated from France, 19 from Israel, 9 from Lebanon, 2 from the USA and 1 each from Turkey, Syria and the Netherlands.

3.2 *Statistical Analysis of Aroona Isoline Data*

All statistical analysis of Aroona isoline quality data was performed using GENSTAT (Release 7.1) software. Flour protein (FP), maximum resistance (R_{max}), extensibility (Ext.) and dough strength (Area) were measured on 990 isoline samples grown at Charlick Experimental Station over a four year period, however not all isolines were grown in all years and this gave rise to an unbalanced data set. Unless stated otherwise, quality data obtained in each of the years was combined prior to statistical analysis

3.2.1 ANOVA of isoline quality data

ANOVA (unbalanced treatment structure) was used to analyse genotypic data so that differences in FP, R_{max}, Ext., and Area in response to N treatment for individual isolines could be determined. For ANOVA, the quality data obtained in each year was not combined, FP was treated as covariate (except for FP determination) and the significance level of the results was calculated relative to the results obtained for Aroona (Appendix II).

eg. ANOVA R_{max} predictions for individual isolines in response to N treatment

```
"Unbalanced Treatment Structure"; BLOCK YEAR+REP+TREAT; TREATMENTS
ISOLINE ID; COVARIATE FP; DELETE [REDEFINE=yes] _ausave; AUNBALANCED
[PRINT=aovtable; FACT=2; FPROB=yes] Rmax; SAVE=_ausave
```

3.2.2 Estimates of variance components on quality parameters

Estimates of variance components on quality parameters were obtained using the Restricted Maximum Likelihood (REML) method which has been recommended for estimating unbiased variances and covariances of unbalanced data (Eagles *et al.*, 2002a). REML analysis (random model) was conducted on the isoline quality data with year, N

treatment, glutenin genotype and interactions treated as random factors. This was done to determine the degree these factors influenced the differences associated with FP, R_{max}, Ext and Area observed in this study (Table 4-1, Chapter 4).

eg. REML estimate of contribution by variance components to differences in R_{max}

```
VCOMPONENTS [FACTORIAL=9] RANDOM=YEAR+TREAT+YEAR.TREAT+ISOLINE
ID+YEAR.ISOLINE ID+TREAT.ISOLINE ID+YEAR.TREAT.ISOLINE ID;
INITIAL=1,1,1,1,1,1,1; CONSTRAINTS=positive,positive,positive,positive,
positive,positive,positive REML [PRINT=model,components;
PSE=differences; MVINCLUDE=*; METHOD=AI; MAXCYCLE=20] Rmax
```

3.2.3 Glutenin allele main effects and additive interactions

3.2.3.1 Allele main effects

Statistical analysis to determine the main effects (without interaction) of *Glu-1* and *Glu-3* alleles was performed using the REML (linear mixed model) method. Mean predictions of R_{max}, Ext. and Area for glutenin alleles were obtained by considering each glutenin locus as a factor and individual alleles as levels. The analysis of each parameter was conducted by treating the factors loci, FP, year, replicate and treatment as fixed while, even though in a common background, isoline was included as a random factor (Table 4-2, Chapter 4).

eg. REML mean predictions of Ext. for *Glu-B3* main effects

```
VCOMPONENTS [FIXED=GluA1+GluB1+GluD1+GluA3+GluB3+
GluD3+FP+(YEAR/REP/TREAT); FACTORIAL=9] RANDOM=ISOLINE ID; INITIAL=1;
CONSTRAINTS=positive; REML [PRINT=model, components, means, waldTests;
PTERMS=GluB3; PSE=differences; MVINCLUDE=*; METHOD=fisher] EXT
```

3.2.3.2 Allele additive interactions

A similar approach using the REML algorithim was also used to investigate two-way glutenin allele interactions present in the double allele (DA) substitution lines. The analysis was conducted as before where loci, FP, year, replicate and treatment were fixed factors and isoline was treated as a random factor. Predictions for Ext., R_{max} and Area

were estimated by fitting the two loci involved in the interaction to the model first, followed immediately by the interaction.

eg. REML predictions of Ext. for two-way interactions between *Glu-A1* and *Glu-B3*

```
VCOMPONENTS [FIXED=GluA1+GluB1+GluD1+GluA3+GluB3+
GluD3+FP+(YEAR/REP/TREAT); FACTORIAL=9] RANDOM=ISOLINE ID; INITIAL=1;
CONSTRAINTS=positive; REML [PRINT=model, components, means, waldTests;
PTERMS=GluA1+GluB3+GluA1.GluB3; PSE=differences; MVINCLUDE=*;
METHOD=fisher] EXT
```

The results obtained from this analysis were entered into tables (Appendix III) and used to compare REML two-way glutenin allele predictions with the REML means predictions for the actual DA substitution lines (Table 4-4, Table 4-5 and Table 4-6, Chapter 4).

REML means predictions of R_{max}, Ext. and Area for actual DA substituted isolines were obtained by treating isoline ID, FP, year, replicate and treatment as fixed factors, no random factors were fitted to the model. The effects of DA substitution were calculated relative to the results obtained for Aroona, the values used to establish significant differences were generated using ANOVA.

eg. REML mean predictions of Ext. for the DA isolines

```
VCOMPONENTS [FIXED=ISOLINE ID+FP+(YEAR/REP/TREAT); FACTORIAL=1] REML
[PRINT=model, components, means, waldTests; PTERMS=ISOLINE ID;
PSE=differences; MVINCLUDE=*; METHOD=AI; MAXCYCLE=20] EXT
```

eg. ANOVA to obtain DA isoline l.s.d. values (P<0.001) for Ext. effects

```
"Unbalanced Treatment Structure"; BLOCK YEAR+REP+TREAT; TREATMENTS
ISOLINE ID; COVARIATE FP; DELETE [REDEFINE=yes] _ausave; AUNBALANCED
[PRINT=aovtable, means; PSE=diff, alllsd; LSDLEVEL=0.1; FACT=1;
FPROB=yes] EXT; SAVE=_ausave
```

Glutenin alleles were considered to interact if the predicted mean for the DA substitution (X^2,Y^2) subtracted from the sum of predicted means for individual allele substitutions (X^1,Y^2 and X^2,Y^1) was significantly different from the predicted mean of the unsubstituted alleles (X^1,Y^1). The effect of substituting two alleles was calculated by subtracting the predicted mean of DA substituted alleles (X^2,Y^2) from the predicted mean of unsubstituted alleles (X^1,Y^1). Where X^2 and Y^2 are the substituted alleles at the X and Y loci, respectively and X^1 and Y^1 are the Aroona alleles.

eg. X^1,Y^1 substituted with X^2,Y^2

	X^1	X^2
Y^1	X^1,Y^1	X^2,Y^1
Y^2	X^1,Y^2	X^2,Y^2

Interaction when $X^2,Y^2 - (X^1,Y^2 + X^2,Y^1) \neq X^1,Y^1$

Effect of DA substitution = $X^2,Y^2 - X^1,Y^1$

3.2.4 Effect of glutenin alleles at different protein levels

The influence of glutenin alleles on R_{max}, Ext. and Area at different protein levels was also predicted using the REML method. For this analysis, the isoline quality data was divided into its respective treatment groups, N nil and N spray, and analysed independently from each other. Again, the glutenin loci, FP, year and replicate were considered as fixed variables while isoline ID was treated as a random factor. To obtain the mean predictions for R_{max}, Ext. and Area, each of the glutenin loci were fitted to the model. The results generated were used to identify which alleles at low protein would be predicted to have at least the same effect on dough quality as others at a higher protein level (Table 4-7, Chapter 4).

eg. REML Ext. predictions for *Glu-A1* alleles in N treatment groups

```
VCOMPONENTS [FIXED=GluA1+GluB1+GluD1+GluA3+GluB3+GluD3+FP+(YEAR/REP);
FACTORIAL=9]\ RANDOM=ISOLINE ID; INITIAL=1; CONSTRAINTS=positive REML
[PRINT=model,components,means,waldTests; PTERMS=GluA1+GluB1+GluD1+GluA3+
GluB3+GluD3; PSE=differences; MVINCLUDE=*; METHOD=AI; MAXCYCLE=20] EXT
```

3.3 *Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis*

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Singh *et al.* (1991) with minor modifications as detailed below. Nomenclature for HMW-GSs followed those of Payne and Lawrence (1983), Marchylo *et al.* (1992) and Gianibelli *et al.* (2002). LMW-GS allelic designations were assigned according to Gupta and Shepherd (1990).

3.3.1 Protein extraction

3.3.1.1 Gliadins

Sample grains were cut in half using a scalpel, the non-germ end was crushed with a hammer to produce a flour sample that was transferred to separate wells in a 96-well extraction block. Extraction of the gliadin fraction was conducted prior to the glutenins by adding 250 µL of 50% (v/v) propan-1-ol to the samples in the extraction blocks. Following 15 min sonication in a sonication bath (Branson 5200, Branson Ultrasonic Corp., Danbury, CT) at 60 °C and 10 min centrifugation (1,968 x g, Sigma 3-10, Sigma-Aldrich, Sydney, NSW) to pellet the flour residue, 50 µL of each supernatant was added to 100 µL of sample buffer (0.02% bromophenol blue; 80 mM Tris-HCl (pH 8.0); 69 mM SDS) in corresponding micro-titre plate wells which were stored at -20 °C prior to electrophoresis. The remaining gliadins present in the flour residue were removed by two subsequent wash steps using 1 mL 50% (v/v) propan-1-ol.

3.3.1.2 Glutenins

The glutenin fraction was extracted from pelleted flour residue using 100 µL of 50% (v/v) propan-1-ol: 80 mM Tris-HCl (pH 8.0) containing 1% (w/v) dithiothreitol (DTT) followed by 30 min sonication in a 60 °C sonication bath. To prevent the reformation of disulfide bonds and improve band resolution, the protein subunits were alkylated for 15 min at 60 °C sonication with 100 µL 50% propan-1-ol: 80 mM Tris-HCl (pH 8.0) containing 1.4% (v/v)

4-vinylpyridine (4-VP). This was followed by 10 min centrifugation (1,968 x g) to pellet the flour residue. Again, a 50 μL aliquot of each reduced and alkylated glutenin protein extract (supernatant) was added to 100 μL of sample buffer in 96-well micro-titre plates and stored at -20 °C until electrophoresis was conducted. Since any particulate matter interferes with SDS-PAGE, prior to running the samples on SDS-PAGE, they were again centrifuged for 10 min (1,968 x g) to ensure that any remaining traces of flour residue were pelleted.

3.3.2 Electrophoresis

3.3.2.1 SDS Gradient PAGE

Electrophoresis apparatus included a Hoeffer SE600 vertical electrophoresis unit (San Francisco, CA.), with 16 x 18 cm glass plates separated by a 1 mm spacer. A discontinuous polyacrylamide gel system of Singh *et al.*, (1991) was modified to employ a 3% stacking gel and a 8-12% acrylamide gradient separating gel with 1.5% cross-linker concentration (bisacrylamide:acrylamide). Gels were loaded with 12 μL of sample and electrophoresis was carried out at approximately 10°C and 40 mA/gel for ≈ 3.5 hr.

3.3.2.2 Uniform SDS-PAGE

As uniform SDS-PAGE was used to identify HMW-GSs it was not necessary to clearly resolve the LMW-GSs, hence the gliadins, which overlap LMW-GSs in SDS-PAGE, were not extracted prior to the glutenins. Electrophoresis apparatus included a Hoeffer SE260 Mighty Small II mini vertical electrophoresis unit, with 10 by 10.5 cm glass plates separated by a 1 mm spacer. A discontinuous polyacrylamide gel system with a 3% stacking gel and a 8.5% acrylamide separating gel with 1.5% cross-linker concentration (bisacrylamide:acrylamide) was employed. Gels were loaded with 8 μL of sample and electrophoresis was carried out at 15 mA per gel for ≈ 3.5 hr with the gels cooled by circulating tap-water.

3.3.3 Visualisation

Gels were stained overnight in Coomassie brilliant blue R-250 (Singh and Shepherd, 1988) and destained in water for 3 hr, aided by the addition of Kimwipes (Kimberley-Clark Corporation, Dallas, Texas) and then equilibrated in 3% glycerol to prevent gels from cracking during drying. The gels were dried between cellophane sheets for at least 3 hr using a Bio-Rad Gel Air Dryer (Bio-Rad Laboratories Inc., Hercules, CA).

3.4 QUALITY TESTING

All quality testing was conducted by AGT (Adelaide, SA) on flour derived from harvested grain cleaned using a Dockage Tester (2 mm screen).

3.4.1 Grain hardness, protein content and moisture content

Prior to flour milling, a small sub-sample of grain (≈ 20 g) was milled using a Laboratory Mill type 3303 (Perten Instruments AB, Sweden) so that grain hardness (PSI), grain protein content and moisture content could be determined indirectly by Near-InfraRed Spectrophotometry (NIR, Technicon InfraAlyzer 450, Bran+Luebbe S.L., Norderstedt, Germany) and expressed on a 13.5 % moisture basis.

3.4.2 Flour milling

The protocol for Buhler milling was based on the standard AACC method 26-20 with minor modification as follows. Based on the NIR results for PSI and moisture content, the grain was conditioned to a specific moisture content relative to the PSI, as defined by the AACC method 26-20 (AACC, 1987), for not less than 18 hrs and not exceeding 24 hrs. For example, a sample with a PSI of 17 was conditioned, by the addition of water, so that it had a 15.9 % moisture content. Tempered grain samples (1500 g) were milled to an approximate 75 % extraction rate using a Buhler mill (Buhler AG, Uzwill, Switzerland) fitted with 44GG (425 μm) top sieves on the break-side, 9xx bottom sieves on the break-

side, and 9xx sieves both top and bottom on the reduction side. Flour protein content (FP) and moisture were measured indirectly by NIR and expressed on a 13.5 % moisture basis.

For Quadrumat Mill derived flours, 320 g of tempered grain was milled using a Brabender Quadrumat Junior Experimental Mill (Brabender® GmbH & Co. KG., Duisburg, Germany) fitted with a 70GG reel sieve (236 μm). The milled sample was subsequently sifted through a 9xx sieve (Simon, Renold Ltd., Wythenshawe, Manchester) so that the particle size of the flour was essentially the same as if it had been milled using a Buhler Mill.

3.4.3 Farinograph

Farinograph analyses were only conducted on Buhler milled flour according to the standard AACC method 54-21 (AACC, 1987) with minor modification. Water absorption (FWA), dough development time (FDDT), dough stability (FDS) and dough breakdown (FDBD) were obtained on a Brabender Farinograph using 50 g (adjusted based on 13.5 % moisture) of flour. Following a dry mix for 1 min, the volume of deionised water (FWA) added (30 °C ± 1 °C) that resulted in a dough with a consistency of 500 BU at FDDT was recorded. FWA provided a guide as to the amount of water required in subsequent dough rheology tests although slight adjustments were necessary to compensate for the addition of salt.

3.4.4 Salt dough farinograph/extensograph

3.4.4.1 Buhler milled flour

Salt dough farinographs/extensograph testing was conducted according to AACC method 54-10 (AACC, 1987) with minor modification. Doughs were prepared by dry mixing ≈ 300 g of flour and 6 g NaCl (table salt) for 1 min in a Brabender Farinograph fitted with a 300 g mixing bowl. Following this, the predetermined volume of deionised water (30 °C ± 1 °C) was added so that after a further 5 min of mixing a final consistency of 500 BU was obtained. Duplicate 150 g dough pieces were formed into balls, rolled and incubated in a

humidity cabinet for 45 min at 30 °C ± 1 °C. Doughs were then stretched at a constant speed until the breaking point was reached using a Brabender Extensograph.

3.4.4.2 Quadrumat Junior Experimental milled flour

This was conducted based on AACC method 54-10 (AACC, 1987) but with significant modification. Dough was prepared by mixing 50 g of flour in a Brabender farinograph with the required volume of 3.16% (w/v) NaCl solution at 30 °C ± 1 °C to give a final consistency of 500 BU after 5 min of mixing. A single 75 g dough piece was formed into a ball, then rolled and incubated in a humidity cabinet for 45 min at 30 °C. Since the Brabender extensograph is designed to operate with a 150 g dough piece, two 37.5 g lead weights were placed on either side of the cradle. Aided by the addition of the lead weights the dough was stretched at a constant speed until the breaking point was reached.

3.4.4.3 Extensograph rheological data

The maximum resistance to extension (R_{max}) in BU was obtained by measuring the maximum height of the extensograph curve (extensogram) while the extensibility (Ext.) in cm was given by the length of the extensogram. The area (cm^2) under the curve reflects both R_{max} and Ext. and is a measure of dough strength.

3.5 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

3.5.1 Reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC analysis of HMW-GSs was based on previously published methods (Marchylo *et al.*, 1989 and 1996) with some minor modifications.

3.5.1.1 Protein extraction

Flour samples were obtained by crushing 5 grains with a hammer. Protein was extracted in 1.5 mL tubes (Eppendorf, Hamburg, Germany) using 500 µL 50% propan-1-ol containing 1% DTT. The samples were placed in a 60 °C sonication bath (Branson 5200) for 30 min

with vortexing at 5 min intervals. This was followed by 10 min centrifugation (10,730 x g, Sigma 201_M) to pellet the flour residue and the supernatant, which contained the extracted protein, was retained. This step was repeated by the addition of another 500 µL 50% propan-1-ol containing 1% DTT aliquot to the previously pelleted flour residue and both supernatants were subsequently combined. To precipitate the HMW-GSs, the propan-1-ol concentration of the protein extract was increased to 60% and then the extract was incubated for 1 hr at 4 °C. Following a 10 min centrifugation (10,730 x g) the supernatant was discarded and the precipitated HMW-GSs were resolubised after the addion of 600 µL of 50% propan-1-ol; 0.2 M Tris-HCl (pH 6.6); 2 M urea; and 1% DTT and sonication for 1 hr in a 60 °C sonication bath. Prior to RP-HPLC, samples were alkylated with 40 µL 50% propan-1-ol containing 71% 4-VP and filtered into 1 mL glass HPLC vials (Waters Corporation, Milford, Massachusetts) using a PVDF 0.45 µm Gelman Acrodisk LC13 Minispike (Pall Corporation, East Hills, NY).

3.5.1.2 RP-HPLC analysis

A Waters HPLC system (600 controller; 486 detector; 717 plus autosampler; and Millennium[32], version 3.2 software) fitted with a C18 column guard (Alltech Associates Australia, Baulkham Hills, NSW) and Zorbax 300SB-C18 column (C18, 300 Å pore size, 3.5 µm particle size, 150mm x 4.6mm id, Agilent Technolgies Austalia, Forest Hill, Victoria) was used. Separations were performed over 70 min at a flow rate of 1 mL/min and at a column temperature of 50 °C. Solvents A and B consisted of water and acetonitrile, respectively, each containing 0.1% trifluoracetic acid (TFA) and sparged with helium at a rate of 20 mL/min. The injection volume was 15 µL and HMW-GSs were eluted using a gradient extending from 26-50% acetonitrile (26% B, 0-3 min; 26-35% B, 3-49.8 min; 35-50% B, 49.8-50 min; 50% B, 50-53.5 min; 50-26% B, 53.5-54 min; 26% B, 54-70 min) while being monitored at a UV detection wavelength of 210nm. RP-HPLC analysis was conducted in duplicate and the mean results reported. Visualisation and

integration of the resulting peaks was performed using Millennium[32] acquisition software. Identification of subunit peaks was based on examples provided in the literature (Marchylo *et al.,* 1989, 1992 and 1996; Sutton, 1991; Margiotta *et al.,* 1993; Lafiandra *et al.,* 1994a and 1994b; Gianibelli *et al.,* 2002).

3.5.2 Size-exclusion high-performance liquid chromatography (SE-HPLC)

3.5.2.1 Protein extraction

Grain samples (≈ 30 seeds) from all of the lines subjected to SE-HPLC analysis in the course of this project were milled using a FQC-200 (Metefem, Budapest, Hungary) micromill and sifted for 30 sec using a 280 μm sieve (Simon). After ≈ 7 days, protein was extracted from the whole meal flour extracts according to Singh *et al.* (1990a and b) and the 2-step method of Gupta *et al.* (1993) with minor modification. For the first extraction, 1 mL of 0.5% SDS in 0.05M phosphate extraction buffer (PEB; pH 6.9) was added to 10 mg (± 1 mg) of sample in 1.5 mL Eppendorf tubes. Samples were vortexed for 30 sec and subjected to 10 min centrifugation (10,730 x g) to pellet SDS-insoluble protein and flour residue. The supernatant (SDS-soluble fraction) was decanted into clean 1.5 mL Eppendorf tubes and set aside.

The remaining pellet was subsequently resuspended in 1 mL PEB and subjected to 30 sec sonication using a Branson sonifier (model B-12) fitted with a 3 mm diameter stepped microtip probe which generated ultrasonic vibrations with a frequency of 22 kHz. Care was taken in positioning the micotip within the Eppendorf tube to ensure that the samples did not froth. The samples were centrifuged (10,730 x g) for 10 min to pellet the flour residue. The supernatant (SDS-insoluble fraction) was transferred to a clean 1.5 mL Eppendorf tube. Prior to SE-HPLC, both extracts were filtered into 1 mL glass HPLC vials (Waters) using a PVDF 0.45 μm Gelman Acrodisk LC13 Minispike and placed in an 80°C waterbath for 2 min to inactivate proteases (Larroque *et al.*, 2000).

3.5.2.2 SE-HPLC analysis

SE-HPLC analysis was performed on a Waters Protein-Pak 300™ column (eg. C18, 300 Å pore size, 3.5 μm particle size, 150mm x 4.6mm id) using a Waters™ 717 plus autosampler, a Waters™ 600 system controller, a Waters™ 486 detector and Millennium[32] (version 3.2 software) for acquisition and reprocessing of data generated from the detector. Separation of both the SDS-soluble and SDS-insoluble fractions was achieved in 40 min by loading 10 μL of sample into an eluent of 50% (v/v) acetonitrile and dH_20 containing 0.1% trifluoracetic acid (TFA) at a flow rate of 0.5 mL/min. Protein was detected by UV absorbance at 214 nm (Batey *et al.*, 1991; Gupta *et al.*, 1992; Stone *et al.*, 1996).

Figure 3-1 provides an example of SE-HPLC chromatograms of both SDS-extractable and SDS-unextractable protein fractions. Addition of the area under the curve of both fractions provided the results for total protein (TP), while %UP (percent of SDS-unextractable protein in total protein) was calculated by dividing the area under the curve of the SDS-unextractable fraction by TP. %PPP (percent of polymeric protein in protein) was determined by summing the polymeric protein peaks of both fractions and dividing this by TP. %FPP is the amount of polymeric protein in flour expressed as a percentage of the total weight of flour which was calculated by multiplying %PPP by FP and then dividing by 100 (Gupta *et al.*, 1992; Bangur *et al.*, 1997). Finally, the %UPP (percent SDS-unextractable polymeric protein in total polymeric protein) was determined by dividing the polymeric protein peak in the SDS-unextractable fraction by the sum of polymeric protein peaks in both SDS-extractable and SDS-unextractable fractions and multiplying this figure by 100 (MacRitchie *et al.*, 1992). To ensure more consistent determination of %UPP between different varieties, the ω-gliadin portion of the chromatograms were included as part of the polymeric protein fraction. This was necessary because of difficulties in discriminating between the end of the first (polymeric protein) and the beginning of the second (ω-gliadin) peaks. Also of note is that some ω-gliadins (D-subunits) possess a

single cysteine residue enabling them to become part of the gluten polymer. The presence of ω-gliadins in the SDS-unextractable fraction would most likely exert a negative effect on dough rheological parameters and bread making quality since they are considered to act as chain terminators (Masci et al., 1993; MacRitchie, 1999)

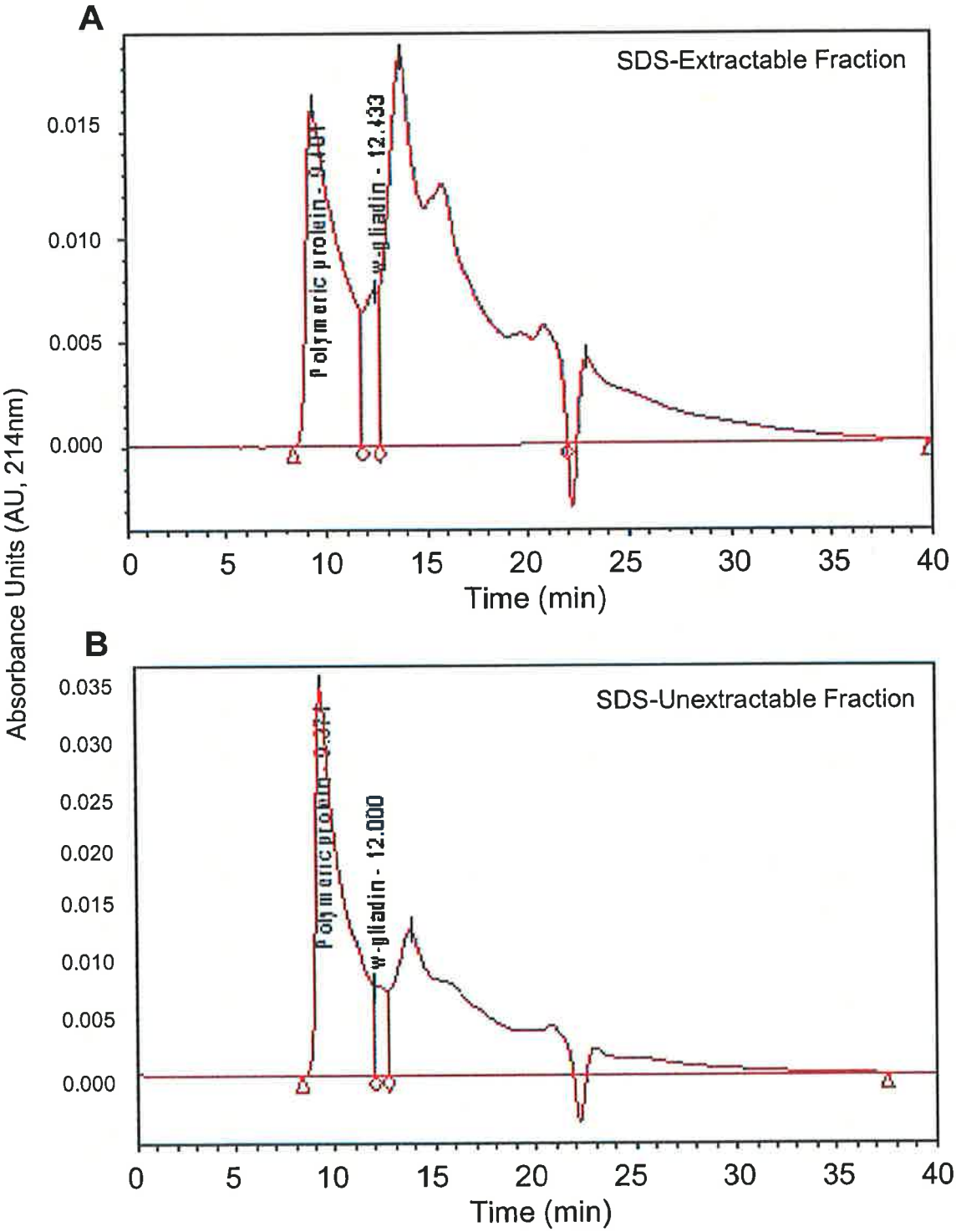


Figure 3-1 SE-HPLC chromatograms of protein extracted from flour derived from the wheat cultivar, Kukri. **A.** SDS-Extractable protein fraction. **B.** SDS-Unextractable protein fraction.

CHAPTER 4

RHEOLOGICAL DOUGH PROPERTIES OF AROONA ISOLINES DIFFERING IN GLUTENIN SUBUNIT COMPOSITION

4.1 INTRODUCTION

The unique cohesive-elastic properties of doughs, made from flour of hexaploid wheat (*Triticum aestivum*), result primarily from the properties of the gluten proteins which make up about 85% of the total protein contained within the endosperm (Lawrence and Shepherd, 1980). These wheat endosperm storage proteins include the monomeric gliadins and the polymeric glutenins. This later group of polymeric proteins, which are the major genotypic determinants of dough strength, consists of HMW-GSs and LMW-GSs encoded at the *Glu-1* and *Glu-3* loci, respectively. Both HMW-GSs and LMW-GSs aggregate through the formation of intra and intermolecular bonds (disulfide and other non-covalent bonds) to form large insoluble complexes that play a key role in the rheological behaviour of flour during bread making. (Benmoussa *et al.*, 2000).

There is considerable allelic variation at each of the homoeologous *Glu-1* and *Glu-3* loci and many studies have shown the effect different HMW-GS alleles have on dough quality parameters. These studies culminated in the construction of an index for the contribution of individual *Glu-1* alleles to dough strength, and has provided an important early-generation selection screen for use in wheat breeding programs (Payne *et al.*, 1987; Shepherd, 1996). However, the glutenin quality index should be expanded to include LMW-GSs.

Systematic use of both HMW-GS and LMW-GS composition by breeders through early generation selection would be greatly facilitated if the contribution of the many different

LMW-GS alleles, the interactions between HMW-GSs and LMW-GSs and the influence of protein content to quality parameters were better understood or quantified. The ideal material for this work would be isogenic lines or biotypes (Gupta *et al.*, 1991)

4.2 Materials and Methods

Aroona isolines (55 lines) comprising a large set of *Glu-1* and *Glu-3/Gli-1* alleles were used to assess the effects different glutenin subunits have on dough rheological parameters and, in conjunction with nitrogen applications, how these effects were influenced by protein content. A list of donor parents in the backcrossing program with Aroona to develop the single allele substitution lines (Aril) is provided in Chapter 3, Table 3-1. Electrophoretic protein banding patterns and a list of all the isolines, including single, double and triple *Glu-1* and *Glu-3/Gli-1* allele substitutions are provided in Appendix I and Appendix II, respectively.

Field trials, protein content determination and salt-dough extensographs on Quadrumat milled flour samples were performed as described in Chapter 3.

4.3 Results and Discussion

4.3.1 SDS Gradient PAGE

Three seeds of each isoline were analysed using SDS Gradient PAGE to confirm glutenin genotype (Appendix I). As expected, the majority of HMW-GSs were readily identifiable while the LMW-GSs were more difficult. As the gliadin (ω- and γ-type) alleles at the *Gli-1* loci are useful genetic markers for dough quality because of their close linkage with *Glu-3* alleles (Redaelli *et al.*, 1997), the gliadin banding patterns assisted in confirming LMW-GS identification (Appendix I).

Contrary to a previous report (Vawser *et al.*, 2002), closer examination of the isolines revealed that the alleles, *Glu-B1u* (7*+8) and *Glu-D3e*, were not present in the allele set. The putative donor of *Glu-B1u*, India 115, was found to contain *Glu-B1b* (7+8). This was not expected since the *Glu-B1b* allele is relatively rare and usually only associated with the variety, Chinese Spring. The misclassification of *Glu-B1b* can be attributed to the similar electrophoretic mobility of protein subunits associated with *Glu-B1b* and *Glu-B1u*. The size difference between subunits *Glu-B1* 7x and *Glu-B1* 7*x is a mere 18bp in the coding region (Butow *et al.*, 2003) and results in the *Glu-B1* 7x subunit being slightly larger (Marchylo *et al.*, 1992) and fractionally less mobile on SDS Gradient PAGE than *Glu-B1* 7*x (Figure 4.1, Lanes 7-9 compared with Lanes 10-12). The presence of the *Glu-B1b* allele in India 115 was also confirmed for this thesis using the PCR molecular marker reported by Butow *et al.* (2003) for *Glu-B1* 7x and by RP-HPLC for the *Glu-B1* 8y subunit (data not shown).

Two of the Aroona isolines possessed unusual banding patterns. One of these appears to be a novel allele encoded at the *Glu-B3* locus, designated *Glu-B3m* and the other, a *Glu-D3* allele not associated with a linked *Gli-D1* ω-gliadin which is subsequently referred to as *Glu-D3a-Gli-D1*$^{-}$.

The single allele substitution of *Glu-B3b* in Aroona with *Glu-B3m* from Bungulla resulted in the Aroona isoline, Aril 27-3. One of the glutenin protein subunits specific to *Glu-B3m* has an electrophoretic mobility less than that for any other LMW-GSs reported in the literature indicating that this is the largest LMW-GS described so far (Figure 4.1, Lanes 10-12 and 17-19). The ω-gliadin that is typically co-inherited with *Glu-B3m* appears to be the same as for *Glu-B3b*. This is not considered to be due to a cross-over event occurring between the *Glu-B3* and *Gli-B1* loci, since Bungulla also expresses the ω-gliadin usually associated with *Glu-B3b* (data not shown).

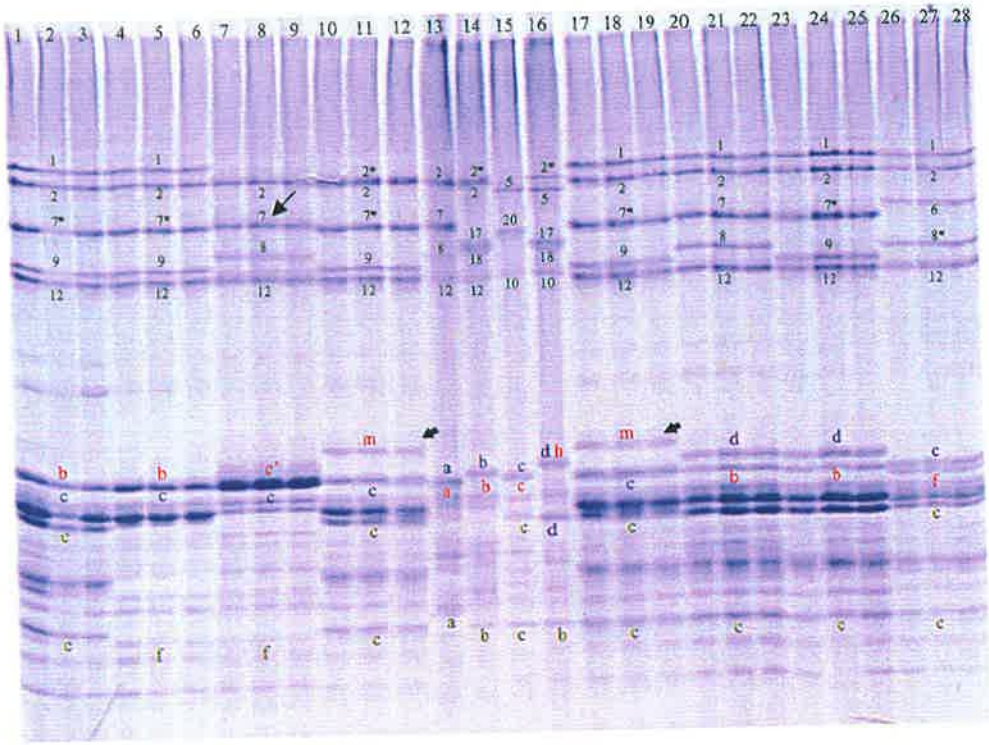


Figure 4-1 SDS Gradient PAGE (8-12%) of *Glu-1* and *Glu-3* glutenin subunits extracted from Aroona isolines. Arrow (lanes 7-9) indicates *Glu-B1* 7x from India 115; Arrowhead (lanes 10-12 and lanes 17-19) indicates LMW-GS specific to *Glu-B3m* in Aroona isolines; blue = *Glu-A3*; red = *Glu-B3*; and green = *Glu-D3*.

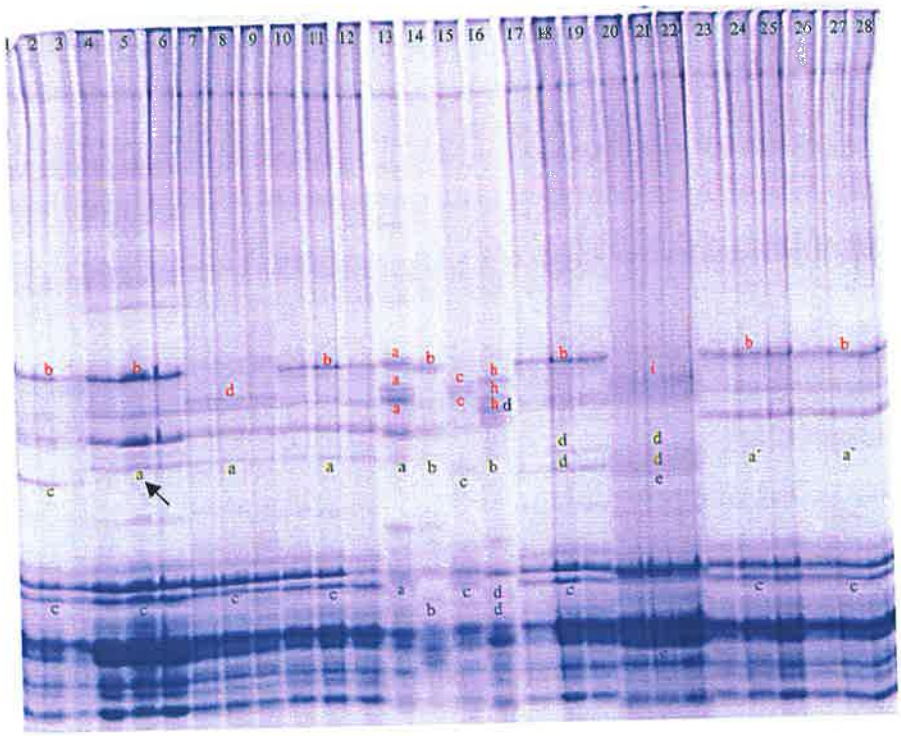


Figure 4-2 SDS Gradient PAGE (8-12%) of gliadin subunits extracted from Aroona isolines. Arrow indicates the ω-gliadin linked to *Glu-D3a* (lanes 4-13) which is missing in *Glu-D3a-Gli-D1*$^{-}$, lanes 23-28. Blue = linked to *Glu-A3*; red = linked to *Glu-B3*; and green = linked to *Glu-D3*.

Glu-D3a-Gli-D1$^-$ in Aril 34-1, previously misclassified as *Glu-D3e* by Vawser *et al.* (2002), was derived from an initial cross between BT2288A (*Glu-D3a-Gli-D1*$^-$) and Aroona (*Glu-D3c*). It has the same LMW-GS SDS Gradient PAGE banding pattern as *Glu-D3a* from the cultivar Chinese Spring but the *Glu-D3a-Gli-D1*$^-$ allele is missing the ω-gliadin subunit that serves as a marker for the *Glu-D3a* allele. This can be seen in Figure 4.2 Lanes 4-12 (*Glu-D3a*) compared with Lanes 23-28 (*Glu-D3a-Gli-D1*$^-$) and appears to be the only way that these alleles can be differentiated using SDS Gradient PAGE.

4.3.2 Glutenin composition

4.3.2.1 Estimates of variance components for quality parameters

REML analysis (random model) was conducted on the isoline quality data incorporating season, treatment and genotype to estimate variance and their relative contribution to differences in FP, R_{max}, Ext. and Area. Variances were expressed as a percentage of the total variation (Table 4-1).

Source of Variation	FP (%)	R_{max} (%)	Ext. (%)	Area (%)
Seasonal	29.6	60.8	18.6	35.7
Treatment	46.3	2.1	50.0	23.5
Genotype	1.0	28.0	2.6	15.4
Interactions	16.1	3.7	17.2	15.7
Residual	7.0	5.4	11.5	9.6

Table 4-1 Relative contribution of variance components to differences in flour protein (FP), maximum resistance (R_{max}), extensibility (Ext.) and dough strength (Area).

As expected, treatment contributed most to the variation observed in FP followed by seasonal factors. Genotype had little effect of FP content. For R_{max}, seasonal factors were the greatest source of variation explaining 60.8% of the total variation. While nitrogen treatment accounted for only 2.1%, the variation associated with genotype was highly significant, contributing 28.0% to the total variation. Genotype constitution in the

population under study has a large effect on variance components. This reduces the relevance of comparisons made between these results and those of other workers but the 28.0% variation in R_{max} attributed to genotype here is in accordance with previous reports (Eagles *et al.,* 2002a). For Ext., nitrogen treatment was the greatest source of variation explaining 50.0% of the total variation, this was followed by seasonal factors which accounted for a further 18.6%, but genotype had little effect (2.6%). These observations confirm the dependence of extensibility on flour protein content rather than glutenin genotype (Gupta *et al.,* 1989 and 1992; Singh *et al.,* 1991b; MacRitchie, 1992 and 1999; Cornish *et al.,*2001). As expected, for dough strength (Area) the contribution of variance components were between those for R_{max} and Ext. Seasonal factors contributed 35.7% to the total variation, nitrogen treatment was responsible for 23.5%, and a further 15.4% could be explained by genotype.

Interactions among variance components explained 16.1%, 3.7%, 17.2% and 15.7% of the difference in FP, R_{max}, Ext. and Area, respectively. Except for R_{max}, year x treatment was responsible for most of the variation associated with interactions. This was not unexpected since :

1) the treatments varied between years; and
2) the reliance of FP, Ext. and to a lesser extent Area, on protein content

For R_{max}, the low year x treatment interaction (0.6%) indicates that the large variation due to seasonal factors was independent from treatment and therefore due to other seasonal factors. These may include moisture stress, heat stress, the timing of heat stress and duration of the growing season. How these factors can affect dough quality is discussed in Chapter 2. Clearly, seasonal factors are very significant and future field trials should also investigate genetic x environment interactions. The residual variation typically describes non-genetic components, such as laboratory variability.

4.3.2.2 Effect of glutenin alleles on quality parameters

In preference to ANOVA, which is unsuitable for analysing large unbalanced data sets (Eagles, pers. comm.)(Table 1, Appendix III), REML analysis was conducted on the data obtained in this study to generate individual allele predictions (without interactions) for the dough quality parameters, R_{max}, Ext. and Area (Table 4-2). This also enabled comparisons to be made between this study and previously published results. Prior to discussing allele main effects it is necessary to mention that the extensograph data, obtained from single pulls of 75g dough pieces, have greater standard errors than the official testing method which requires duplicate pulls using 150g dough pieces (Cornish, pers. comm.). This would decrease the number of significantly different results obtained for the glutenin alleles in this study.

For *Glu-A1* alleles, the null allele, *Glu-A1c* had a significant negative influence on all three dough rheological parameters. This result was expected and confirms the findings of previous studies (Payne *et al.*, 1987; Eagles *et al.*, 2002a). The effect of *Glu-A1p* on dough quality has not previously been reported, and was not significantly different from *Glu-A1a* and *Glu-A1b*. The ranking of *Glu-A1* alleles observed in this study was *Glu-A1a* = *p* = *b* > *c* for R_{max}, Ext. and Area

At the *Glu-B1* locus, glutenin allele ranking for R_{max} and Area were similar, *Glu-B1i* = *b* = *c* > *d* = *a,* which broadly agrees with the *Glu-1* quality score developed by Payne *et al.* (1987) where *Glu-B1a* and *Glu-B1d* both received the same low rank, while *Glu-B1i* and *Glu-B1b* received a higher *Glu-1* quality score than *Glu-B1c*. In contrast, the results from this study showed that although *Glu-B1i* exerted the greatest positive effect, there was no significant difference between the *Glu-B1i*, *Glu-B1b* and *Glu-B1c* alleles. For Ext., REML predictions showed that *Glu-B1a*, *Glu-B1c* and *Glu-B1i* were significantly more extensible than *Glu-B1d*. However, comparisons with findings here for *Glu-B1b* with those obtained

Allele	HMW-GS	R_{max} (Bu)		Ext. (cm)		Area (cm^2)		Allele	R_{max} (Bu)		Ext. (cm)		Area (cm^2)	
		Mean	Effect	Mean	Effect	Mean	Effect		Mean	Effect	Mean	Effect	Mean	Effect
Glu-A1								***Glu-A3***						
a	1	194 ^{A}	+13	14.8 ^{A}	+0.4	54.2 ^{A}	+5.6	*a*	173 ^{A}	−3	14.8 ^{A}	+0.4	48.7 ^{A}	+0.1
b	2*	178 ^{A}	+2	14.7 ^{A}	+0.3	49.8 ^{A}	+1.2	*b*	193 ^{A}	+17	14.7 ^{A}	+0.3	54.8 ^{A}	+6.2
c	null	146 ^{B}	−30	13.7 ^{B}	−0.7	38.8 ^{B}	−9.9	*c*	178 ^{A}	+2	14.6 ^{A}	+0.2	49.6 ^{A}	+1.0
p	3*	187 ^{A}	+11	14.5 ^{A}	+0.1	51.8 ^{A}	+3.2	*d*	200 ^{A}	+24	14.7 ^{A}	+0.3	55.4 ^{A}	+6.8
								e	134 ^{B}	−42	13.6 ^{B}	−0.8	35.7 ^{B}	−13.0
								f	178 ^{A}	+2	14.0 ^{AB}	−0.4	47.7 ^{A}	−1.0
Glu-B1								***Glu-B3***						
a	7*	134 ^{B}	−42	14.7 ^{A}	+0.3	38.7 ^{B}	−10.0	*a*	153 ^{DE}	−23	13.9 ^{D}	−0.5	39.1 ^{DE}	−9.6
b	7+8	192 ^{A}	+16	14.4 ^{AB}	0.0	52.0 ^{A}	+3.4	*b*	205 ^{AB}	+29	14.1 ^{CD}	−0.3	53.7 ^{ABC}	+5.1
c	7*+9	190 ^{A}	+14	14.6 ^{A}	+0.2	52.7 ^{A}	+4.1	*c*	120 ^{E}	−56	14.0 ^{D}	−0.4	33.2 ^{E}	−15.5
d	6+8*	151 ^{B}	−25	13.8 ^{B}	−0.6	39.8 ^{B}	−8.9	*d*	180 ^{BCD}	+4	15.2 ^{AB}	+0.8	53.3 ^{ABC}	+4.7
i	17+18	214 ^{A}	+38	14.7 ^{A}	+0.3	60.0 ^{A}	+11.4	*f*	167 ^{CD}	−9	14.8 ^{B}	+0.4	47.9 ^{BC}	−0.8
								g	222 ^{A}	+46	14.8 ^{B}	+0.4	60.7 ^{A}	+12.1
								h	169 ^{B}	−7	14.0 ^{D}	−0.4	44.1 ^{CD}	−4.6
								i	170 ^{B}	−6	15.5 ^{A}	+1.1	50.7 ^{ABC}	+2.1
								m	199 ^{ABC}	+23	14.7 ^{BC}	+0.3	55.2 ^{AB}	+6.6
Glu-D1								***Glu-D3***						
a	2+12	160 ^{B}	−16	14.6 ^{A}	+0.2	45.3 ^{B}	−3.4	*a*	158 ^{C}	−18	14.6 ^{A}	+0.2	44.5 ^{BC}	−4.2
b	3+12	158 ^{B}	−18	14.6 ^{A}	+0.2	45.9 ^{B}	−2.8	*a-Gli-D1*$^{-}$	217 ^{A}	+41	14.7 ^{A}	+0.3	59.1 ^{A}	+10.2
d	5+10	251 ^{A}	+75	14.0 ^{A}	−0.4	63.2 ^{A}	+14.6	*b*	147 ^{C}	−29	14.2 ^{A}	−0.2	40.2 ^{C}	−8.5
f	2.2+12	135 ^{B}	−41	14.6 ^{A}	+0.2	40.2 ^{B}	−8.5	*c*	174 ^{BC}	−2	14.2 ^{A}	−0.2	46.4 ^{BC}	−2.3
								d	160 ^{C}	−16	14.8 ^{A}	+0.4	48.0 ^{BC}	−0.7
								f	201 ^{AB}	+25	14.2 ^{A}	−0.2	53.8 ^{AB}	+5.2

Table 4-2 REML mean predictions and allele main effects (without interactions) for R_{max}, Ext. and Area. FP treated as a co-variate. Results followed by the same letter are not significantly different ($P \leq 0.05$) from each other. Effects relative to mean of all alleles (R_{max} = 176 BU; Ext. = 14.4 cm; Area = 48.7 cm^2); red = negative effect; blue = postitive effect. Gene frequencies and summary data presented in Appendix II and III.

in previous studies should be treated with caution as more than one *Glu-B1* allele encodes subunits with similar electrophoretic mobilities to *Glu-B1b* namely, the *Glu-B1u*, *Glu-B1al,* and *Glu-B1ak* alleles. Consequently, these alleles may have been misclassified in earlier work.

Predictions reported here for alleles at the *Glu-D1* locus were consistent with the *Glu-1* quality score of Payne *et al.* (1987). The superiority of the *Glu-D1d* is well established and reflected in the quality score of Payne *et al.* (1987). In this study it was significantly better that *Glu-D1a*, *Glu-D1b* and *Glu-D1f* for both R_{max} and Area with the alleles ranked in the following order: *Glu-D1d* > *a* = *b* = *f*. While the *Glu-D1d* allele had a positive influence on R_{max} and Area an inverse relationship between *Glu-D1d* and Ext. was observed. Although not significant in this study, a decrease in extensibility associated with the presence of *Glu-D1d* has been reported by Cornish *et al.* (2001) and Eagles *et al.* (2002a and 2004).

The results obtained in this study for LMW-GS alleles are consistent with previous reports (Cornish *et al.*, 2001; Eagles *et al.*, 2002a and 2004; Liu *et al.*, 2005). At the *Glu-A3* locus, *Glu-A3e* (null) exerted a significant negative influence on R_{max} and Area and, although not significant, tended to be less extensible than the other *Glu-A3* alleles included in this study. The effect of *Glu-A3* alleles on dough rheology, in decreasing order, was *Glu-A3d* = *b* = *c* = *a* = *f* > *e* for R_{max} and Area. Differences in Ext. between *Glu-A3f* and *Glu-A3e* were not significant.

Predicted values for the main effects of *Glu-B3* alleles on dough rheological parameters showed the largest degree of significant variation. *Glu-B3g*, *Glu-B3b* and the newly described allele *Glu-B3m* resulted in similar high values for R_{max}, whereas the alleles which

exerted a negative impact on R_{max} included *Glu-B3a* and *Glu-B3c*. For R_{max}, the *Glu-B3* alleles were ranked as follows, *Glu-B3* (*g* = *b* = *m*); (*b* = *m* = *d* = *h* = *i*); (*m* = *d* = *f*); (*d* = *f* = *a*); (*a* = *c*). With regard to extensibility, two alleles, *Glu-B3i* and *Glu-B3d*, had a positive influence while *Glu-B3g*, *Glu-B3f* and *Glu-B3m* were associated with average extensibility. Allele *Glu-B3b*, typically associated with high dough strength, *Glu-B3c*, *Glu-B3h* and *Glu-B3a* were linked with lower extensibility. The ranking of alleles for Ext. was *Glu-B3* (*i* = *d*); (*d* = *f* = *g* = *m*); (*m* = *b*); (*b* = *c* = *h* = *a*). For dough strength (Area), the *Glu-B3* alleles ranked as follows, *Glu-B3* (*g* = *m* = *b* = *d* = *i*); (*m* = *b* = *d* = *i* = *f*); (*b* = *d* = *i* = *f* = *h*); (*h* = *a*); (*a* = *c*). A positive effect of *Glu-B3g* on gluten strength was also reported by Eagles *et al.* (2002a) and Liu *et al.* (2005) however, other studies suggested that the effect of *Glu-B3g* on quality attributes was similar to *Glu-B3h* (Eagles *et al.*, 2004; Bekes *et al.*, 2004). Possible explanations include inconsistent effects of *Glu-3* loci on R_{max} and Ext. across environments (Ma *et al.*, 2004) and/or the absence of the *Glu-B3g* linked ω-gliadin (eg. Millewa; Appendix I, Figure 6, Lanes 17-19) which was investigated in this study, compared with *Glu-B3g* cultivars that do express a linked ω-gliadin (eg. Angas; not shown). Since *Glu-B3g* is being actively selected in Australian wheat breeding programs it seems imperative to determine whether there is a difference in quality associated with *Glu-B3g* ± ω-gliadin.

Even though the effect of *Glu-D3* on quality traits is reported to be less important compared to other loci (Gupta *et al.*, 1994; Eagles *et al.*, 2002a; Branlard *et al.*, 2003) significant variation was observed in this study and is most likely due to the greater number of *Glu-D3* alleles included here. Furthermore, difficulties associated with the identification of *Glu-D3* alleles could have led to misclassification and masked the effects associated with them in earlier work. More research is required to ensure reliable and routine identification of *Glu-D3* alleles. The effect on R_{max} of the subunits encoded at the *Glu-D3* locus were ranked *Glu-D3* (*a-GliD1*$^{-}$ = *f*); (*f* = *c*); (c = *d* = *a* = *b*) while for Area the

ranking was *Glu-D3* (*a-GliD1*$^-$ = *f*); (*f* = *a* = *c* =*d*); (*a* = *c* = *d* = *b*). There were no significant differences between predicted extensibilities associated with the different *Glu-D3* alleles, although *Glu-D3a*, *Glu-D3a-GliD1*$^-$ and *Glu-D3d* tended to be more extensible than *Glu-D3b*, *Glu-D3c* and *Glu-D3f*.

Rankings for the overall effect of *Glu-1* and *Glu-3* alleles on dough rheological parameters (Table 4-3) highlight the importance of both HMW-GSs and LMW-GSs. The four glutenin alleles which positively contributed to R_{max} and Area are *Glu-D1d*, *Glu-B3g*, *Glu-D3a-Gli-D1*$^-$ and *Glu-B1i*. The reported frequencies of *Glu-D1d*, *Glu-B3g* and *Glu-B1i* in southern Australian wheat breeding programs was 51.2%, 5.2% and 24.9%, respectively (Eagles *et al.*, 2002a) while the frequency of *Glu-D3a-Gli-D1*$^-$ is unknown as it has not been previously described.

Table 4-3 also shows that to increase both R_{max} and Ext., *Glu-B3g* would be better than *Glu-D1d* and highlights the need to ensure that when *Glu-D1d* is selected in a breeding program, the alleles present at the other glutenin loci are those that are likely to positively contribute to extensibility. *Glu-B3i* and *Glu-B3d* with frequencies of 0.2% and 2.6%, respectively (Eagles *et al.*, 2002a) had the greatest positive effect on Ext. These alleles are very difficult to distinguish from each other on SDS Gradient PAGE (Appendix I, Figure 10, Lanes 20-22, *Glu-B3d*; Lanes 26-28, *Glu-B3i*) but in this study it was observed that *Glu-B3i* was associated with brown glume colour (*Rg1*), located a genetic distance of 3.5cM from the *Glu-B3* locus (Unrau 1950; Payne *et al.*, 1986). Interestingly, *Glu-B3g* and *Glu-B3c* are also linked with the allele for brown glumes (Shepherd, pers. comm.). The *Glu-B3c*, *Glu-A3e* and *Glu-B1e* alleles are the basis of the low quality in the Federation derivatives which dominated south eastern Australia for most of the late 19th and early 20th centuries.

Maximum Resistance (R_{max}, BU)		Extensibility (Ext., cm)		Dough Strength (Area, cm^2)	
Allele	**Prediction**	**Allele**	**Prediction**	**Allele**	**Prediction**
Glu-D1d	251 ± 19.5	*Glu-B3i*	15.5 ± 0.34	*Glu-D1d*	63.2 ± 5.20
Glu-B3g	222 ± 20.0	*Glu-B3d*	15.2 ± 0.34	*Glu-B3g*	60.7 ± 5.43
Glu-D3a-Gli-D1$^-$	217 ± 21.0	*Glu-B3g*	14.8 ± 0.34	*Glu-B1i*	60.0 ± 4.92
Glu-B1i	214 ± 18.3	*Glu-A1a*	14.8 ± 0.30	*Glu-D3a-Gli-D1*$^-$	59.1 ± 5.76
Glu-B3b	205 ± 20.0	*Glu-A3a*	14.8 ± 0.34	*Glu-A3d*	55.4 ± 5.67
Glu-D3f	201 ± 21.0	*Glu-D3d*	14.8 ± 0.38	*Glu-B3m*	55.2 ± 5.43
Glu-A3d	200 ± 21.2	*Glu-B3f*	14.8 ± 0.34	*Glu-A3b*	54.8 ± 5.67
Glu-B3m	199 ± 20.0	*Glu-B1i*	14.7 ± 0.30	*Glu-A1a*	54.2 ± 4.41
Glu-A1a	194 ± 16.4	*Glu-D3a-Gli-D1*$^-$	14.7 ± 0.38	*Glu-D3f*	53.8 ± 5.76
Glu-A3b	193 ± 21.2	*Glu-A3d*	14.7 ± 0.34	*Glu-B3b*	53.7 ± 5.43
Glu-B1b	192 ± 18.3	*Glu-B3m*	14.7 ± 0.34	*Glu-B3d*	53.3 ± 5.43
Glu-B1c	190 ± 18.3	*Glu-A3b*	14.7 ± 0.34	*Glu-B1c*	52.7 ± 4.92
Glu-A1p	187 ± 16.4	*Glu-A1b*	14.7 ± 0.30	*Glu-B1b*	52.0 ± 4.92
Glu-B3d	180 ± 20.0	*Glu-B1a*	14.7 ± 0.30	*Glu-A1p*	51.8 ± 4.41
Glu-A1b	178 ± 16.4	*Glu-B1c*	14.6 ± 0.30	*Glu-B3i*	50.7 ± 5.43
Glu-A3c	178 ± 21.2	*Glu-A3c*	14.6 ± 0.34	*Glu-A1b*	49.8 ± 4.41
Glu-A3f	178 ± 21.2	*Glu-D1b*	14.6 ± 0.31	*Glu-A3c*	49.6 ± 5.67
Glu-D3c	174 ± 21.0	*Glu-D1a*	14.6 ± 0.31	*Glu-A3a*	48.7 ± 5.67
Glu-A3a	173 ± 21.2	*Glu-D3a*	14.6 ± 0.38	*Glu-D3d*	48.0 ± 5.76
Glu-B3i	170 ± 20.0	*Glu-D1f*	14.6 ± 0.31	*Glu-B3f*	47.9 ± 5.43
Glu-B3h	169 ± 20.0	*Glu-A1p*	14.5 ± 0.30	*Glu-A3f*	47.7 ± 5.67
Glu-B3f	167 ± 20.0	*Glu-B1b*	14.4 ± 0.31	*Glu-D3c*	46.4 ± 5.76
Glu-D3d	160 ± 21.0	*Glu-D3f*	14.2 ± 0.38	*Glu-D1b*	45.9 ± 5.20
Glu-D1a	160 ± 19.5	*Glu-D3c*	14.2 ± 0.38	*Glu-D1a*	45.3 ± 5.20
Glu-D1b	158 ± 19.5	*Glu-D3b*	14.2 ± 0.38	*Glu-D3a*	44.5 ± 5.76
Glu-D3a	158 ± 21.0	*Glu-B3b*	14.1 ± 0.34	*Glu-B3h*	44.1 ± 5.43
Glu-B3a	153 ± 20.0	*Glu-D1d*	14.0 ± 0.31	*Glu-D3b*	40.2 ± 5.76
Glu-B1d	151 ± 18.3	*Glu-A3f*	14.0 ± 0.34	*Glu-D1f*	40.2 ± 5.20
Glu-D3b	147 ± 21.0	*Glu-B3c*	14.0 ± 0.34	*Glu-B1d*	39.8 ± 4.92
Glu-A1c	146 ± 16.4	*Glu-B3h*	14.0 ± 0.34	*Glu-B3a*	39.1 ± 5.43
Glu-D1f	135 ± 19.5	*Glu-B3a*	13.9 ± 0.34	*Glu-A1c*	38.8 ± 4.41
Glu-B1a	134 ± 18.3	*Glu-B1d*	13.8 ± 0.31	*Glu-B1a*	38.7 ± 4.92
Glu-A3e	134 ± 21.2	*Glu-A1c*	13.7 ± 0.30	*Glu-A3e*	35.7 ± 5.67
Glu-B3c	120 ± 20.0	*Glu-A3e*	13.6 ± 0.30	*Glu-B3c*	33.2 ± 5.43

Table 4-3 Overall ranking of REML means predictions for the main *Glu-1* and *Glu-3* allele effects (all years, treatment groups combined) on maximum resistance (R_{max}), extensibility (Ext.) and dough strength (Area). Flour protein (FP) was treated as a co-variate; Significance level $P \leq 0.05$.

4.3.2.3 Additive effects of glutenin alleles on quality parameters

An additive gene effect occurs when the combined allele effect is equal to the sum of the individual allele main effects, while a genetic interaction is apparent when the combined allele effect is greater or less than the sum of the individual allele main effects. To identify allelic interactions among the double allele (DA) substitution isolines comparisons were made between REML predictions for two-way glutenin allele interactions (Appendix III) and the REML predicted means for actual DA lines (data not shown). The way in which the statistical analysis was conducted is outlined in Chapter 3.

The actual effects of double allelic subustitutions in the DA lines were calculated relative to the mean prediction for the recurrent parent, Aroona. Significant differences were estimated based on the l.s.d. obtained using ANOVA for each individual DA lines compared to Aroona (data not shown). For R_{max} (Table 4-4) a large number (18 out of 21) of the DA isolines had predictions highly significantly different from Aroona. Twelve of the double allele substitutions in these lines resulted in a decrease in R_{max} while six lines obtained higher R_{max} predictions. In four of the six lines with higher R_{max}, *Glu-D1a* was substituted with *Glu-D1d*. Isolines that were not significantly different from Aroona included DA 2* A3d, DA 2.2+12 A3d and DA 5+10 B3c.

For Ext. (Table 4-5) the number of significantly different DA isoline predictions, compared to Aroona, was less than for R_{max} (7 out of 21 were highly significant $P<0.001$ and a further 5 significant at the $P<0.01$ or $P<0.05$ lsd levels) and with the exception of DA 7+8 B3d and DA B3d D3a, all were negative. The significant increase in Ext. observed for DA 7+8 B3d and DA B3d D3a is in accordance with what was reported previously for *Glu-B3d*. A similar result would also have been likely for DA isolines, if they were produced, that contained the *Glu-B3i* allele. In eleven of the DA isolines, the glutenin alleles that were substituted did not significantly alter Ext. predictions.

Isoline	Aroona	Substitution	REML estimate on the basis of the DA substitution lines			REML prediction on the basis of additive effects of individual substitutions			Interaction [d]
	X^1,Y^1	X^2,Y^2	Mean R_{max} (BU)	Effect [a]		Mean R_{max} (BU ± ave. SED) [b] X^1,Y^1	X^2,Y^2	Effect [c]	
DA 2* A3d	*Glu-A1a, Glu-A3c*	*Glu-A1b, Glu-A3d*	226	+5		196	202 ± 21.9	+6	-1
DA 2* A3e	*Glu-A1a, Glu-A3c*	*Glu-A1b, Glu-A3e*	164	-57	***	196	136 ± 21.9	-60	+3
DA 2* B3d	*Glu-A1a, Glu-B3b*	*Glu-A1b, Glu-B3d*	174	-47	***	223	182 ± 22.6	-41	-6
DA 2* B3m	*Glu-A1a, Glu-B3b*	*Glu-A1b, Glu-B3m*	196	-25	***	223	201 ± 22.6	-22	-3
DA 2.2+12 A3d	*Glu-D1a, Glu-A3c*	*Glu-D1f, Glu-A3d*	218	-3		162	160 ± 23.5	-2	-1
DA 3* D3a-Gli-D1$^-$	*Glu-A1a, Glu-D3c*	*Glu-A1p, Glu-D3a-Gli-D1$^-$*	261	+40	***	191	227 ± 23.0	+36	+4
DA 5+10 A3d	*Glu-D1a, Glu-A3c*	*Glu-D1d, Glu-A3d*	323	+102	***	162	275 ± 23.5	+113	-11
DA 5+10 A3e	*Glu-D1a, Glu-A3c*	*Glu-D1d, Glu-A3e*	275	+54	***	162	209 ± 23.5	+47	+7
DA 5+10 B3c	*Glu-D1a, Glu-B3b*	*Glu-D1d, Glu-B3c*	222	+1		189	194 ± 24.1	+5	-4
DA 5+10 B3h	*Glu-D1a, Glu-B3b*	*Glu-D1d, Glu-B3h*	276	+55	***	189	244 ± 24.1	+55	0
DA 6+8* B3f	*Glu-B1c, Glu-B3b*	*Glu-B1d, Glu-B3f*	155	-66	***	221	142 ± 24.1	-77	+11
DA 7+8 A3d	*Glu-B1c, Glu-A3c*	*Glu-B1b, Glu-A3d*	244	+23	***	194	216 ± 23.6	+24	-1
DA 7+8 A3e	*Glu-B1c, Glu-A3c*	*Glu-B1b, Glu-A3e*	173	-48	***	194	150 ± 23.6	-42	-6
DA 7+8 B3a	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3a*	168	-53	***	221	169 ± 24.1	-50	-3
DA 7+8 B3c	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3c*	156	-65	***	221	135 ± 24.1	-84	+19
DA 7+8 B3d	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3d*	236	+15	**	221	196 ± 24.1	-23	+38
DA 7+8 B3h	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3h*	185	-36	***	221	185 ± 24.1	-34	-2
DA 7* A3d	*Glu-B1c, Glu-A3c*	*Glu-B1a, Glu-A3d*	185	-36	***	194	158 ± 23.6	-34	-2
DA B3d D3a	*Glu-B3b, Glu-D3c*	*Glu-B3d, Glu-D3a*	137	-84	***	202	162 ± 25.7	-40	-44
DA null A3a	*Glu-A1a, Glu-A3c*	*Glu-A1c, Glu-A3a*	191	-30	***	196	143 ± 21.9	-53	+23
DA null D3a	*Glu-A1a, Glu-D3c*	*Glu-A1c, Glu-D3a*	156	-65	***	191	128 ± 23.0	-63	-2

[a] Effect relative to Aroona (mean R_{max} = 221 ± 7.4 BU); * P<0.05, ** P<0.01, *** P<0.001 (red = negative effect; blue = positive effect)

[b] From Appendix III, Table 1(a)

[c] Effect = X^2,Y^2 - X^1,Y^1 where X^1,Y^1 are the unsubstituted alleles in Aroona and X^2,Y^2 are the substituted alleles present in DA isolines

[d] Interaction = Effect of substituted alleles in DA isolines – Effect of substituted alleles from REML 2-way predictions

Table 4-4 R_{max} comparison between mean REML predictions for DA isolines and REML 2-way allele interaction predictions.

Isoline	Aroona	Substitution	REML estimate on the basis of the DA substitution lines			REML prediction on the basis of additive effects of individual substitutions			
	X^1,Y^1	X^2,Y^2	Mean Ext. (cm)	Effect [a]		Mean Ext. (cm ± ave. SED) [b] X^1,Y^1	X^2,Y^2	Effect [c]	Interaction [d]
DA 2* A3d	*Glu-A1a, Glu-A3c*	*Glu-A1b, Glu-A3d*	14.6	-0.2		15.0	14.9 ± 0.39	-0.1	-0.1
DA 2* A3e	*Glu-A1a, Glu-A3c*	*Glu-A1b, Glu-A3e*	13.7	-1.1	***	15.0	13.9 ± 0.39	-1.1	0
DA 2* B3d	*Glu-A1a, Glu-B3b*	*Glu-A1b, Glu-B3d*	15.6	+0.8		14.5	15.4 ± 0.38	+0.9	-0.1
DA 2* B3m	*Glu-A1a, Glu-B3b*	*Glu-A1b, Glu-B3m*	15.3	+0.5		14.5	14.9 ± 0.38	+0.4	+0.1
DA 2.2+12 A3d	*Glu-D1a, Glu-A3c*	*Glu-D1f, Glu-A3d*	15.0	+0.2		14.8	14.8 ± 0.41	0	-0.2
DA 3* D3a-Gli-D1$^-$	*Glu-A1a, Glu-D3c*	*Glu-A1p, Glu-D3a-Gli-D1$^-$*	15.0	+0.2		14.6	14.7 ± 0.43	+0.1	-0.1
DA 5+10 A3d	*Glu-D1a, Glu-A3c*	*Glu-D1d, Glu-A3d*	14.0	-0.8	***	14.8	14.2 ± 0.41	-0.6	-0.2
DA 5+10 A3e	*Glu-D1a, Glu-A3c*	*Glu-D1d, Glu-A3e*	13.0	-1.8	***	14.8	13.2 ± 0.41	-1.6	-0.2
DA 5+10 B3c	*Glu-D1a, Glu-B3b*	*Glu-D1d, Glu-B3c*	14.2	-0.6	**	14.2	13.5 ± 0.40	-0.7	+0.1
DA 5+10 B3h	*Glu-D1a, Glu-B3b*	*Glu-D1d, Glu-B3h*	13.9	-0.9	***	14.2	13.6 ± 0.40	-0.6	-0.3
DA 6+8* B3f	*Glu-B1c, Glu-B3b*	*Glu-B1d, Glu-B3f*	14.5	-0.3		14.3	14.2 ± 0.40	-0.1	-0.2
DA 7+8 A3d	*Glu-B1c, Glu-A3c*	*Glu-B1b, Glu-A3d*	14.3	-0.5	*	14.8	14.7 ± 0.41	-0.1	-0.4
DA 7+8 A3e	*Glu-B1c, Glu-A3c*	*Glu-B1b, Glu-A3e*	13.5	-1.3	***	14.8	13.6 ± 0.41	-1.2	-0.1
DA 7+8 B3a	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3a*	14.2	-0.6		14.3	13.9 ± 0.40	-0.4	-0.2
DA 7+8 B3c	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3c*	14.1	-0.7	**	14.3	13.9 ± 0.40	-0.4	-0.3
DA 7+8 B3d	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3d*	16.0	+1.2	***	14.3	15.1 ± 0.40	+0.8	+0.4
DA 7+8 B3h	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3h*	14.2	-0.6	**	14.3	14.0 ± 0.40	-0.3	-0.3
DA 7* A3d	*Glu-B1c, Glu-A3c*	*Glu-B1a, Glu-A3d*	14.6	-0.2		14.8	14.9 ± 0.41	-0.1	-0.1
DA B3d D3a	*Glu-B3b, Glu-D3c*	*Glu-B3d, Glu-D3a*	15.5	+0.7	*	13.9	15.3 ± 0.45	+1.4	-0.7
DA null A3a	*Glu-A1a, Glu-A3c*	*Glu-A1c, Glu-A3a*	13.6	-1.2	***	15.0	14.1 ± 0.39	-0.9	-0.3
DA null D3a	*Glu-A1a, Glu-D3c*	*Glu-A1c, Glu-D3a*	14.4	-0.4		14.6	13.8 ± 0.43	-0.8	+0.4

[a] Effect relative to Aroona (mean Ext. = 14.8 ± 0.30 cm); * P<0.05, ** P<0.01, *** P<0.001 (red = negative, blue = positive)

[b] From Appendix III, Table 2(a)

[c] Effect = X^2,Y^2 - X^1,Y^1 where X^1,Y^1 are the unsubstituted alleles in Aroona and X^2,Y^2 are the substituted alleles present in DA isolines

[d] Interaction = Effect of substituted alleles in DA isolines – Effect of substituted alleles from REML 2-way predictions

Table 4-5 Ext. comparison between mean REML predictions for DA isolines and REML 2-way allele interaction predictions.

Isoline	Aroona	Substitution	REML estimate on the basis of the DA substitution lines			REML prediction on the basis of additive effects of individual substitutions			
	X^1,Y^1	X^2,Y^2	Mean Area (cm^2)	Effect [a]		Mean Area (cm^2 ± ave. SED) [b] X^1,Y^1	X^2,Y^2	Effect [c]	Interaction [d]
DA 2* A3d	*Glu-A1a, Glu-A3c*	*Glu-A1b, Glu-A3d*	59.4	-0.6		55.2	56.5 ± 5.82	+1.3	-1.9
DA 2* A3e	*Glu-A1a, Glu-A3c*	*Glu-A1b, Glu-A3e*	42.2	-17.8	***	55.2	36.9 ± 5.82	-18.3	+0.5
DA 2* B3d	*Glu-A1a, Glu-B3b*	*Glu-A1b, Glu-B3d*	51.3	-8.7	*	59.3	54.4 ± 6.04	-4.9	-3.8
DA 2* B3m	*Glu-A1a, Glu-B3b*	*Glu-A1b, Glu-B3m*	56.3	-3.7		59.3	56.3 ± 6.04	-3.0	-0.7
DA 2.2+12 A3d	*Glu-D1a, Glu-A3c*	*Glu-D1f, Glu-A3d*	60.3	+0.3		46.3	46.9 ± 6.23	+0.6	-0.3
DA 3* D3a-Gli-D1$^-$	*Glu-A1a, Glu-D3c*	*Glu-A1p, Glu-D3a-Gli-D1$^-$*	70.4	+10.4	***	51.9	62.2 ± 6.12	+10.3	+0.1
DA 5+10 A3d	*Glu-D1a, Glu-A3c*	*Glu-D1d, Glu-A3d*	80.2	+20.2	***	46.3	70.0 ± 6.23	+23.7	-3.5
DA 5+10 A3e	*Glu-D1a, Glu-A3c*	*Glu-D1d, Glu-A3e*	63.5	+3.5		46.3	50.3 ± 6.23	+4.0	-0.5
DA 5+10 B3c	*Glu-D1a, Glu-B3b*	*Glu-D1d, Glu-B3c*	56.4	-3.6		50.4	47.7 ± 6.42	-2.7	-0.9
DA 5+10 B3h	*Glu-D1a, Glu-B3b*	*Glu-D1d, Glu-B3h*	66.9	+6.9	***	50.4	58.7 ± 6.42	+8.3	-1.4
DA 6+8* B3f	*Glu-B1c, Glu-B3b*	*Glu-B1d, Glu-B3f*	44.5	-15.5	***	57.8	39.1 ± 6.45	-18.7	+3.2
DA 7+8 A3d	*Glu-B1c, Glu-A3c*	*Glu-B1b, Glu-A3d*	62.0	+2.0		53.7	58.8 ± 6.25	+5.1	-3.1
DA 7+8 A3e	*Glu-B1c, Glu-A3c*	*Glu-B1b, Glu-A3e*	42.0	-18.0	***	53.7	39.1 ± 6.25	-14.6	-3.4
DA 7+8 B3a	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3a*	44.3	-15.7	***	57.8	42.5 ± 6.45	-15.3	-0.4
DA 7+8 B3c	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3c*	40.5	-19.5	***	57.8	36.5 ± 6.45	-21.3	+1.8
DA 7+8 B3d	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3d*	70.9	+10.9	***	57.8	56.6 ± 6.45	-1.2	+12.1
DA 7+8 B3h	*Glu-B1c, Glu-B3b*	*Glu-B1b, Glu-B3h*	47.6	-12.4	***	57.8	47.5 ± 6.45	-10.3	-2.1
DA 7* A3d	*Glu-B1c, Glu-A3c*	*Glu-B1a, Glu-A3d*	49.4	-10.6	***	53.7	45.4 ± 6.25	-8.3	-2.3
DA B3d D3a	*Glu-B3b, Glu-D3c*	*Glu-B3d, Glu-D3a*	44.0	-16.0	***	51.4	49.1 ± 6.93	-2.3	-13.7
DA null A3a	*Glu-A1a, Glu-A3c*	*Glu-A1c, Glu-A3a*	47.0	-13.0	***	55.2	38.9 ± 5.82	-16.3	+3.3
DA null D3a	*Glu-A1a, Glu-D3c*	*Glu-A1c, Glu-D3a*	42.6	-17.4	***	51.9	34.7 ± 5.82	-17.2	-0.2

[a] Effect relative to Aroona (mean Ext. = 60.0 ± 2.78 cm^2); * P<0.05, ** P<0.01, *** P<0.001 (red = negative, blue = positive)

[b] From Appendix III, Table 3(a)

[c] Effect = X^2,Y^2 - X^1,Y^1 where X^1,Y^1 are the unsubstituted alleles in Aroona and X^2,Y^2 are the substituted alleles present in DA isolines

[d] Interaction = Effect of substituted alleles in DA isolines – Effect of substituted alleles from REML 2-way predictions

Table 4-6 Area comparison between mean REML predictions for DA isolines and REML 2-way allele interaction predictions.

Results obtained in relation to Area (Table 4-6) were similar to those obtained for R_{max} and Ext. A number of DA isolines (15 out of 21) produced doughs that were significantly different in strength from Aroona with eleven being weaker and four stronger.

Keeping in mind that the standard errors in this study were quite large, no significant glutenin gene epistaic interactions were observed for either R_{max}, Ext. or Area in this data set. Although, the differences in the effects associated with two DA isolines, DA 7+8 B3d and DA B3d D3a, and the effects predicted based on individual additive main effects of the substituted alleles were consistently high and approaching significance for R_{max}, Area and to a lesser extent Ext. (Table 4-4, Table 4-6 and Table 4-5, respectively). Interestingly both these DA isolines contained the *Glu-B3d* allele and the possible epistatic effects were positive for DA 7+8 B3d and negative for DA B3d D3a. The glutenin alleles present in DA 7+8 B3d have been confirmed by SDS Gradient PAGE analysis of the flour samples used for the dough rheological tests (one from each year, data not shown). Clearly, confidence in any conclusions regarding *Glu-B3d* interactions would require further investigation, ideally with a more balanced data set and smaller standard errors.

Investigations into the double allele substitutions present in the DA isolines included in this study revealed that genetic interactions between glutenin alleles was not a major factor that influenced REML predictions for R_{max}, Ext. and Area. However, the important contribution of additive glutenin gene effects to these dough parameters was confirmed showing that alleles associated with strong dough properties (moderate to high R_{max}, high extensibility), confer even greater resistance and extensibility when combined. For example, the similar R_{max} results obtained for the isoline DA 5+10 A3d and REML predictions for additive individual allele main effects. The effect of substituting *Glu-D1a* and *Glu-A3c* (221 BU) with *Glu-D1d* and *Glu-A3d* (323 BU) to produce the DA 5+10 A3d isoline resulted in an increase in R_{max} of +102 BU, an effect that closely resembles the sum

of REML predictions for the individual main effects of *Glu-D1d* (+91 BU) and *Glu-A3d* (+22 BU) of +113 BU. These results also indicate that REML can be used to reliably predict allele main effects in unbalanced data sets, highlighting the importance of software programs that can be used to predict dough quality attributes in progeny arrays from specific crosses, such as 'CrossPredictor' jointly developed by the Molecular Plant Breeding CRC and The University of Queensland (Eagles *et al.*, 2002a and b).

As the quality of data entered into statistical programs to analyse the relationship between glutenin alleles and dough quality improves, the number of significant gene interactions decrease (Eagles, pers. comm.). The absence of significant genetic interaction between glutenin alleles in this study may be due to the large standard errors or that the effects associated with the majority of glutenin alleles are simply additive effects.

4.3.3 Flour protein content and glutenin composition

4.3.3.1 Response of isolines to Nitrogen application

Several million tonnes of wheat are produced in Australia each year with a protein content below 10% which is the minimum protein requirement for the AWB market classification, Australian Premium White (APW). Nitrogen (N) was applied to the Aroona isolines in field trials so that harvested grain varied substantially in protein content. This was undertaken to ascertain whether the effects of glutenin alleles were influenced by protein content. This is especially important in southern and western wheat growing regions where some of the wheat produced has only low to medium protein content.

Appendix II provides a list of the 55 isolines included in this study, the isolines grown in each year as well as the differences in mean FP, R_{max}, Ext. and Area between the two nitrogen treatment groups. The mean ANOVA predictions for R_{max}, Ext. and Area are also presented. There was a significant positive response in mean FP to N application in all

years with the response positively correlated ($R^2 = 0.95$, n = 4) with the amount of nitrogen applied. The mean FP between treatment groups was highly significant ($P \leq 0.001$) with the differences being 1.11% (7.63-8.74%) in 2000, 3.85% (7.54-11.39%) in 2001, 3.14% (9.88-13.02%) in 2002 and 1.5% (7.77-9.27%) in 2003. The intention was to apply more nitrogen in 2003 than the 15 g/m^2 urea applied over a three week period (5 g/m^2 urea per week), but an abrupt end to the growing season, due to limited rainfall, prevented subsequent applications. FP in the untreated group was only significantly different in 2002 (mean FP = 9.88%), the year in which only 253mL of rainfall was received between the months of April to October compared to > 320mL for the other years (mean FP = 7.54% to 7.77%). With regard to individual isolines, 13% of the lines grown in 2000, where the least amount of nitrogen was applied (10 g/m^2 urea), did not have a significant mean FP response. However, in the other years, a significant difference was evident in all lines for mean FP between treatment groups, at least at the $P \leq 0.01$ level. The lack of a significant response in some of the lines in 2000 is most likely due to the application of only 10 g/m^2 of urea in that year indicating that to reliably obtain a significant FP response, at least 15 g/m^2 of urea should be applied post anthesis.

The isolines responded positively and significantly to nitrogen treatment in all years for all the rheological parameters measured. There were fewer significant increases in R_{max} (24%) than there were for Ext., where 85% of the lines showed significant increases in extensibility in response to treatment (Appendix II). For dough strength (Area), a significant, positive response to increased FP was evident in 69% of the isolines (Appendix III). This can be directly attributed to the important contribution FP has on extensibility and the positive impact increased extensibility has on dough strength.

Figure 4-3 shows the relationship between maximum resistance or extensibility and dough strength at different protein levels. For the majority of end products, the rheological

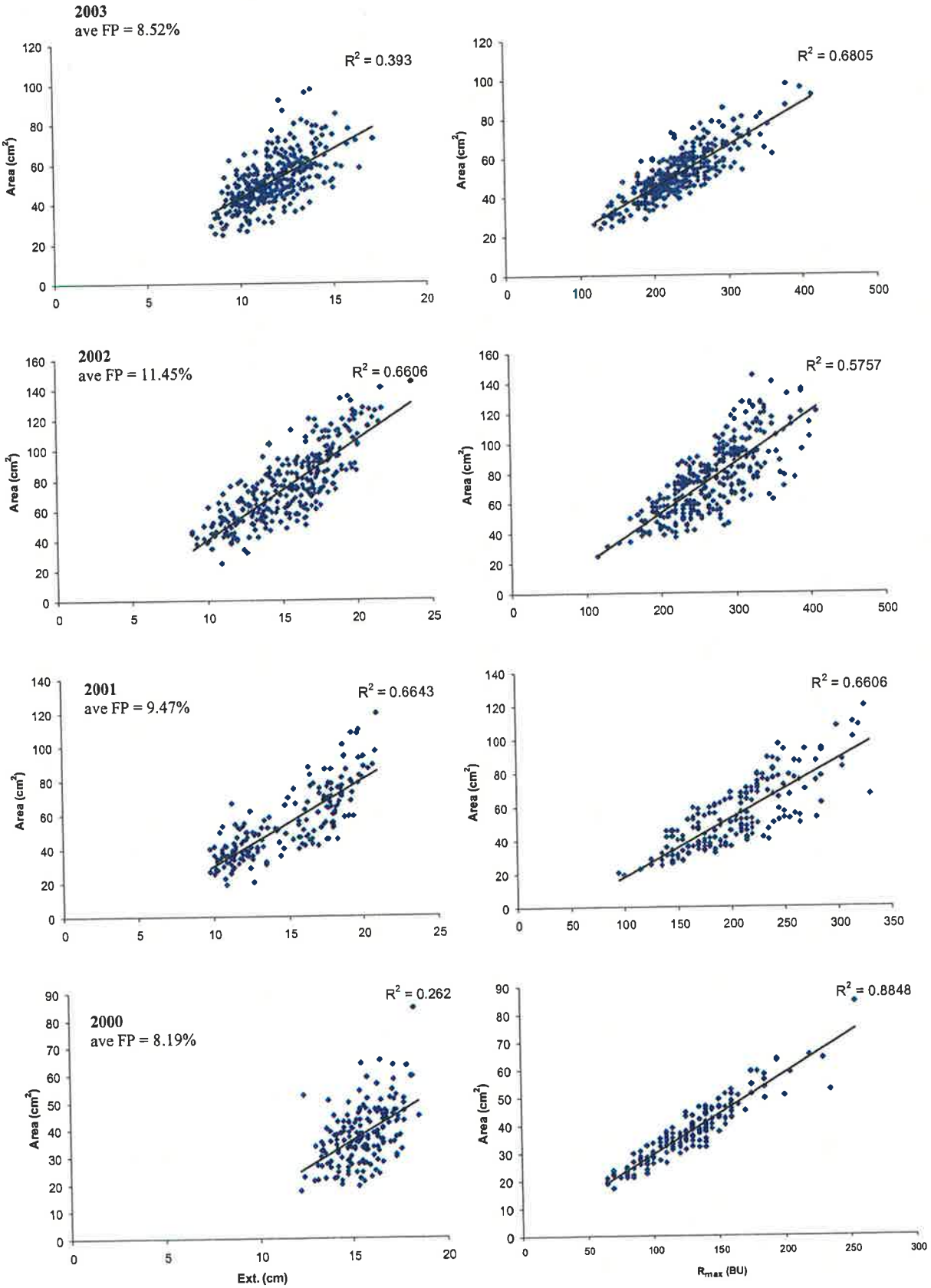


Figure 4-3 Linear regression analysis between dough strength (Area) and extensibility (Ext.) or maximum resistance (R_{max}) for individual isolines in 2000, 2001, 2002 and 2003.

properties of a dough need to be balanced, possessing moderate to high R_{max} and high Ext. Since dough strength, as determined by extensograph area under the curve, is a parameter reported to describe the balance of a dough (Gupta *et al.*, 1989; Simmonds, 1989; Sapirstein and Fu, 1998) incorrect assumptions could be made regarding the contribution of R_{max} and Ext. to dough strength. For example, the possible expectation that correlations between R_{max} versus Area and Ext. versus Area would be similar. It was observed here that R^2 values for these correlations were higher for R_{max} in years where FP was low, 8.52% in 2003 and 8.19% in 2000. While, in 2001 and 2002, where the protein content was 9.47% and 11.45%, respectively, the correlations for R_{max} and Ext. with Area were similar, suggesting that only at higher FP levels, the contribution of R_{max} and Ext. to dough strength are in essence, equal (Figure 4-3). This is most likely due to the pronounced effect FP has on Ext. but could be influenced by the difference in FP between N treatment groups in years where FP was lower, 1.11% in 2000 and 1.5% in 2003 compared to 3.85% in 2001 and 3.14% in 2002. The possibility that this phenomenon reflects errors in small-scale extensograph measurements of extensibility using a single 75g dough piece should also be considered. From a breeding perspective, Ext. evaluations of potential varieties for bread making purpose, if conducted using a 75g dough piece, should be conducted on samples with a FP greater than 10%, the minimum FP considered suitable for pan-bread manufacture. This may explain why the AWB only considers quality data generated from Buhler milled flour and traditional extensograph tests when classifying a new variety for release.

4.3.3.2 Effect of N treatment and glutenin alleles on dough rheology

The difference in protein content between N nil and N spray treatment groups was 2.4% with average values of 8.3% and 10.7%, respectively and corresponded to AWB ASW and APW market classifications. R_{max}, Ext. and Area means predictions and main effects for individual glutenin alleles obtained for both treatment groups using REML analysis are

Allele FP (%)	R_{max} (BU) N nil = 8.3; N spray = 10.7				Ext. (cm) N nil = 8.3; N spray = 10.7				Area (cm^2) N nil = 8.3; N spray = 10.7			
Glu-A1	Effect [a]		Effect [b]		Effect [c]		Effect [d]		Effect [e]		Effect [f]	
a (1)	**192**	+ 14	198	+ 22	13.2	+ 0.3	16.6	+ 0.4	46.4	+ 3.5	62.7	+ 7.6
b (2*)	**182**	+ 4	175	− 1	13.2	+ 0.3	16.4	+ 0.2	45.0	+ 2.1	55.2	+ 0.1
c (null)	151	− 28	**144**	− 32	12.2	− 0.7	15.4	− 0.8	34.8	− 8.1	43.7	− 11.5
p (3*)	**190**	+ 11	188	+ 11	13.0	+ 0.1	16.3	+ 0.1	45.4	+ 2.5	58.9	+ 3.8
	Ave SED = 15.7				Ave SED = 0.31				Ave SED = 4.2			
Glu-B1												
a (7*)	139	− 40	**131**	− 45	13.0	+ 0.1	16.5	+ 0.3	34.3	− 8.6	43.9	− 11.2
b (7+8)	**197**	+ 18	190	+ 15	12.8	− 0.1	16.2	0	46.2	+ 3.3	58.6	+ 3.5
c (7*+9)	**187**	+ 8	195	+ 19	13.0	+ 0.1	16.4	+ 0.2	44.8	+ 1.9	61.4	+ 6.3
d (6+8*)	155	− 24	149	− 27	12.6	− 0.3	15.4	− 0.8	36.3	− 6.6	43.7	− 11.4
i (17+18)	**216**	+ 37	214	+ 38	13.1	+ 0.2	16.4	+ 0.2	52.9	+ 10.0	67.9	+ 12.8
	Ave SED = 18.0				Ave SED = 0.35				Ave SED = 4.8			
Glu-D1												
a (2+12)	160	− 19	**162**	− 14	13.0	+ 0.1	16.3	+ 0.1	39.6	− 3.3	51.7	− 3.4
b (3+12)	163	− 16	**156**	− 20	12.9	0	16.5	+ 0.3	40.3	− 2.6	52.4	− 2.8
d (5+10)	**251**	+ 73	252	+ 77	12.4	− 0.5	15.8	− 0.4	**55.4**	+ 12.5	71.6	+ 16.5
f (2.2+12)	140	− 39	**133**	− 42	13.2	+ 0.3	16.2	0	36.3	− 6.6	**44.8**	− 10.3
	Ave SED = 18.3				Ave SED = 0.35				Ave SED = 4.9			
Glu-A3												
a	174	− 5	172	− 4	13.3	+ 0.4	16.5	− 0.3	42.8	− 0.1	55.1	0
b	**195**	+ 17	194	+ 18	12.9	0	17.0	+ 0.8	46.1	+ 3.2	64.3	+ 9.2
c	**180**	+ 2	178	+ 2	13.1	+ 0.2	16.3	+ 0.1	44.0	+ 1.1	55.9	+ 0.8
d	**205**	+ 27	197	+ 21	13.1	+ 0.2	16.5	+ 0.3	49.5	+ 6.6	61.9	+ 6.8
e	136	− 42	**137**	− 39	12.2	− 0.7	15.3	− 0.9	30.5	− 12.4	41.9	− 13.2
f	**181**	+ 2	177	+ 1	12.9	0	15.4	− 0.8	44.4	+ 1.5	51.7	− 3.4
	Ave SED = 18.5				Ave SED = 0.36				Ave SED = 4.9			
Glu-B3												
a	149	− 30	***151****	− 25	12.4	− 0.5	15.4	− 0.8	34.1	− 8.8	44.1	− 11.0
b	***206****	+ 28	206	+ 31	12.4	− 0.5	15.8	− 0.4	47.0	+ 4.1	61.1	+ 6.0
c	120	− 59	**122**	− 54	12.6	− 0.3	15.4	− 0.8	29.9	− 13.0	**37.3**	− 17.8
d	**176**	− 3	189	+ 13	13.3	+ 0.4	17.0	+ 0.8	44.3	+ 1.4	63.7	+ 8.6
f	**171**	− 8	165	− 11	13.4	+ 0.5	16.1	+ 0.1	43.7	− 0.8	52.6	− 2.5
g	***232****	+ 53	216	+ 40	12.8	− 0.1	16.8	+ 0.6	**52.7**	+ 9.8	69.4	+ 14.3
h	**181**	+ 2	162	− 14	12.5	− 0.4	15.5	− 0.7	41.4	− 1.5	47.6	− 7.5
i	**175**	− 3	168	− 8	13.7	+ 0.8	17.3	+ 1.1	45.5	+ 2.6	56.9	+ 1.8
m	**198***	+ 20	203	+ 27	12.9	0	16.4	+ 0.2	47.7	+ 4.8	63.4	+ 8.3
	Ave SED = 19.3				Ave SED = 0.39				Ave SED = 5.2			
Glu-D3												
a	158	− 20	**157**	− 19	13.0	+ 0.1	16.3	+ 0.1	38.4	− 4.5	50.3	− 4.8
a-Gli-D1[-]	**224**	+ 46	210	+ 34	12.9	0	16.6	+ 0.4	51.9	+ 9.0	66.3	+ 11.2
b	144	− 34	**150***	− 26	12.8	− 0.1	15.7	− 0.5	34.7	− 8.2	45.9	− 9.2
c	175	− 4	173	− 3	12.7	− 0.2	15.9	− 0.3	40.7	− 2.7	52.1	− 3.0
d	172	− 7	**161**	− 15	13.5	+ 0.6	16.4	+ 0.2	46.1	+ 3.2	53.9	− 1.2
f	**198***	+ 20	204	+ 29	12.4	− 0.5	16.1	− 0.1	45.5	+ 2.6	62.2	+ 7.1
	Ave SED = 20.5				Ave SED = 0.43				Ave SED = 5.6			

Effect relative to REML overall mean of [a] 179 BU; [b] 176 Bu; [c] 12.9 cm; [d] 16.2 cm; [e] 42.9 cm^2; [f] 55.1 cm^2

Table 4-7 R_{max}, Ext. and Area predictions for *Glu-1* and *Glu-3* alleles at different FP. levels. **Bold** and/or * denotes predictions for alleles significantly ($P \leq 0.05$) higher at 8.3% FP than alleles with 10.7% FP. Red = positive effect and blue = negative effect.

presented in Table 4-7. Significant increases in FP and Ext. were obtained for all glutenin alleles when compared to each other in response to N treatment. This was as expected since N treatment was shown to have a large effect on these variables (Table 4-1). Area predictions for most of the glutenin alleles suggested a significant increases in response to N treatment. Those that did not increase significantly include *Glu-B1d*, *Glu-D1f*, *Glu-A3f*, *Glu-B3a*, *c*, *f* and *h* and *Glu-D3d*. No glutenin alleles were associated with a significant response in R_{max} to N treatment. Again, this was expected based on the low contribution N treatment had to the total variance for R_{max} in this study (2.1%, Table 4-1).

The R_{max} associated with some alleles at low FP was significantly greater ($P \leq 0.05$) than others at high FP. These include *Glu-A1a*, *b* and *p* compared with *Glu-A1c*; *Glu-B1b*, *c* and *i* compared with *Glu-B1a*; and *Glu-D1d* at low FP with *Glu-D1a*, *b* and *f* at high FP. For the LMW-GS alleles, R_{max} predictions for *Glu-A3b*, *c*, *d* and *f* were significantly higher than for *Glu-A3e* at high FP. Similarly, *Glu-B3b*, *g*, and *m* compared to *Glu-B3a*, while *Glu-B3c* had a lower R_{max} at high FP than *Glu-B3b*, *d*, *f*, *g*, *h*, *i* and *m* at low FP. At the *Glu-D3* locus, *Glu-D3a*, *b* and *d* had a significantly lower R_{max} at greater FP than *Glu-D1a-Gli-D1*$^{-}$ and predictions for *Glu-D3f* indicate that this allele would convey a higher R_{max} at lower FP than *Glu-D3b* at higher protein levels. For dough strength (Area), the results showed that *Glu-D1d* > *Glu-D1f* and *Glu-B3g* > *Glu-B3c* where *Glu-D1d* and *Glu-B3g* were predicted to be stronger at low FP compared to *Glu-D1f* and *Glu-B3c* at high FP. By contrast, the extensibility of all alleles in the glutenin allele set was significantly less ($P \leq 0.05$) at low FP than at high FP (Table 4-7).

The main effects calculated for the glutenin alleles at the different FP levels suggest that gene effects are similar irrespective of FP, or at least when the effects at an FP of 8.3% are compared with effects of the same allele at 10.7% FP. Only two alleles, *Glu-A3b* and *Glu-A3f*, received significantly different values for main effects on Ext. between treatment

groups and the difference was positive for *Glu-A3b* (+ 0.8 cm) and negative for *Glu-A3f* (− 0.8 cm) (Table 4-7). Interestingly, substitution with *Glu-A3b* did not influence Ext. at low FP but the effect conveyed at a higher FP resulted in a higher Ext. prediction than the Aroona base, this combined with *Glu-A3b's* positive contribution to R_{max} at both FP levels would make it a desirable allele in breeding programs.

4.4 CONCLUSION

In this study a set of Aroona isolines differing in glutenin genotype was used to investigate the effect these genes have on dough rheological parameters. Never before has such a comprehensive range of *Glu-1* and *Glu-3* alleles been studied in a common background. Whilst the effects of a large proportion of HMW-GSs on dough quality have already been established and the *Glu-1* quality score has assisted in the selection of desirable parents in wheat breeding programs, the results here serve to increase our understanding of glutenin alleles, particularly by determining the effects of various LMW-GSs. Additive gene effects, possible interactions and the influence of glutenin alleles at different FP on dough rheological parameters were also investigated.

Seasonal factors had a large impact on R_{max}, Ext. and Area, and foliar application of nitrogen after anthesis significantly increased FP in all years explaining more than half of the variation in Ext. While glutenin alleles had little effect on Ext. (2.6%) they were the source of 28.0% and 15.4% of the total variation related to R_{max} and Area, respectively (Table 4.1). No significant epistatic interactions between glutenin loci were identified in the DA substitution lines when compared with two-way REML allele predictions although R_{max} and Area estimates for DA 7+8 B3d and DA B3d D3a appeared to be close to significance and require futher investigation (Table 4-4 and Table 4-6). The importance of additive allele main effects rather than interactions was highlighted, confirming previous

reports that the results of combinations of alleles are equal to the sum of the individual allele main effects. Clearly, the alleles present at each of the *Glu-1* and *Glu-3* loci can have a large combined effect on dough properties and suitability for specific end products, and that flour quality can be improved more efficiently by selecting for both *Glu-1* and *Glu-3* subunits than for either group alone (Gupta *et al.*, 1994a).

Even though the variation in extensibility resulting from different glutenin alleles was small, some significant differences were obtained suggesting that allelic variation can provide useful genetic improvement in extensibility. For high extensibility, the REML predictions indicated that the glutenin allelic combination of *Glu-A1a*, *Glu-B1i* or *a*, *Glu-D1a, b* or *f*, *Glu-A3a*, *Glu-B3i* and *Glu-D3d* would be best suited. In addition, the rheological data obtained here showed that at low protein, extensograph curve area correlates very highly and positively with R_{max}, while at higher protein levels Area correlates highly and significantly with both R_{max} and Ext., reflecting the balance that exists between these two important quality parameters at higher protein levels. This suggests that for meaningful research into the relationship between glutenin alleles and bread making potential, especially for Ext., the wheat samples analysed, if conducted using a single 75g dough piece, should possess at least 10% FP.

Of the alleles tested in this study, the allelic combination that would provide the highest maximum resistance (R_{max}) and dough strength (Area) was shown to be *Glu-A1a*, *Glu-B1i*, *Glu-D1d*, *Glu-A3d*, *Glu-B3g* and *Glu-D3a-Gli-D1*$^{-}$. However, combining these stronger alleles may result in an overstrong dough. It was also evident that the effect on R_{max}, Ext. and Area associated with glutenin alleles were similar at different protein levels however, only Ext. predictions were significantly greater at higher FP (10.7% FP compared to 8.3% FP) for all the glutenin alleles investigated in this study. There were no significant differences in R_{max} predictions between N treatment groups for any of the glutenin alleles

highlighting the important relationship between glutenin genes and R_{max}.

The difference in R_{max} associated with glutenin alleles substantiates varietal classification as well as FP as criteria for entry into AWB market grades. In addition, recent changes to AWB receival standards means that a cultivar classified as APW will not be automatically downgraded to ASW if the protein content is less than 10%. On the 30th August 2005, a base rate of $188.00/t and $179.00/t at 10% FP applied for both the APW and ASW grades, respectively. Premiums were then either reduced or increased based on actual FP content, screenings and moisture content (Australian Wheat Board International Ltd, 2005).

Significant improvement in the dough quality of Australian wheat varieties could be achieved by increasing the frequency of favorable alleles. The frequency in Australian breeding programs of the HMW-GS alleles associated with high R_{max} is relatively high, *Glu-A1a* (1) = 59.4%, *Glu-B1i* (17+18) = 21.8% and *Glu-D1d* (5+10) = 50.2% (Eagles *et al.*, 2004). By comparison, frequencies reported by Eagles *et al.* (2004) for *Glu-A3d* and *Glu-B3g* in Australian germplasm were as low as 6.0% and 1.9%, respectively, with the frequencies of *Glu-B3i*, *Glu-B3d*, *Glu-D3a-Gli-D1*$^{-}$ and *Glu-D3f* even lower. From a breeding and selection perspective, these alleles are readily identifed, particularly when the glutenin composition of the parents is known. Admittedly, *Glu-B3i* and *Glu-B3d* are difficult to distinguish from each other on SDS Gradient PAGE but both these alleles were shown to have a positive influence on dough extensibility. As discussed earlier, identification of LMW-GS alleles encoded by the *Glu-D3* locus is also difficult. It is therefore fortunate that *Glu-D3* alleles associated with high quality, *Glu-D3f* and previously undescribed *Glu-D3a-Gli-D1*$^{-}$, can be distinguished from each other and from other *Glu-D3* alleles present in Australian germplasm using SDS Gradient PAGE.

The results presented here describe the relationship between glutenin alleles and some of the functional properties of wheat flour dough. They also augment previous findings that were based on fewer alleles (Payne *et al.*,1987 and 1990; Gupta *et al.*,1989 and 1994a; Carillo *et al.*,1990; Kolster *et al.*,1992; Cornish *et al.*,2001) and show that alleles which exert positive additive effects can be accumulated to improve wheat quality as it relates to bread making potential.

CHAPTER 5

OVER-EXPRESSION OF HMW GLUTENIN SUBUNIT *GLU-B1* 7X IN HEXAPLOID WHEAT VARIETIES

5.1 INTRODUCTION

Typically, identification of HMW-GSs is based on their "apparent" M_r derived from relative mobilities (R_m) in SDS-PAGE and, in the past, has been considered relatively unambiguous. However, closer examination of HMW-GSs has revealed that different HMW-GSs can appear to have the same R_m in SDS-PAGE analysis. Consequently, there has been incorrect identification and subsequent misleading characterisation of some HMW-GS alleles (Sutton, 1991; Gianibelli *et al.*, 2001). In particular, *Glu-B1b* (7+8), *Glu-B1u* (7*+8), *Glu-B1ak* (7*+8*) and *Glu-B1al* (7+8*) are four different alleles at the same locus with similar electrophoretic mobilities but contrasting effects on quality (Sutton, 1991; Marchylo, 1992; Gianibelli *et al.*, 2001). In the context of bread making quality, the large positive influence of the *Glu-B1al* allele is quite unique and of the most interest. The presence of *Glu-B1al* typically results in a significant increase in the expression of the 1Bx7 subunit. This over-expression, which results in an increase in dough strength, is considered to be the reason for the positive relationship between the *Glu-B1al* allele and bread making quality (Marchylo *et al.*, 1992; Rooke *et al.*, 1999; Gianibelli *et al.*, 2002; Lukow *et al.*, 2002; Juhasz *et al.*, 2003b; Butow *et al.*, 2003).

In the present study, which has been published (Vawser and Cornish, 2004; refer Appendix IV), reversed-phase high-performance liquid chromatography (RP-HPLC) was used to identify germplasm from around the world that contains the *Glu-B1al* allele. These results enabled the identification of the most likely source of the *Glu-B1al* allele in hexaploid wheat cultivars as well as investigation of the elution and expression profiles of HMW-GSs associated with different *Glu-1* alleles.

5.2 *Materials and Methods*

Uniform SDS-PAGE (8.5%) and RP-HPLC were used to investigate HMW-GS composition and elution/expression profiles, respectively. Details of grain samples analysed and protocols are provided in Chapter 3.

5.3 *Results and Discussion*

5.3.1 *Uniform SDS-PAGE*

Uniform SDS-PAGE, which separates proteins according to size, has been an efficient method to identify HMW-GSs, enabling reliable routine differentiation between numerous alleles on a single gel. Figure 5-1 provides an example of the HMW-GS banding patterns associated with the most common *Glu-1* alleles. Subunits encoded on chromosome 1D, *Glu-D1a* (2+12) and *Glu-D1d* (5+10) are readily identified using uniform SDS-PAGE (Figure 5-1, compare lane 6 with lane 5, respectively). *Glu-A1a* (1), *Glu-A1b* (2*) and *Glu-A1c* (null) can also be distinguished from each other (Figure 5-1, lanes 4, 5 and 9, respectively) but sometimes the 1Ax2* can be obscured by the presence of subunit 1Dx2 from *Glu-D1a* (Figure 5-1, lanes 5 and 6, respectively) making it difficult to distinguish *Glu-A1b* (lane 5) from *Glu-A1c* (lane 9). The *Glu-B1* alleles, *Glu-B1e* (20; lane 1), *Glu-B1i* (17+18; lane 2), *Glu-B1f* (13+16; lane 3), *Glu-B1d* (6+8*; lane 4) and *Glu-B1c* (7*+9; lane 5) can all be easily identified using uniform SDS-PAGE. However, the *Glu-B1* alleles, *Glu-B1u* (7*+8), *Glu-B1ak* (7*+8*), *Glu-B1al* (7+8*) and *Glu-B1b* (7+8) have both x- and y-type subunits with similar electrophoretic mobilities (Figure 5-1, refer lanes 6, 7, 8 and 9, respectively). Although subunit 1Bx7* is reported to be slightly more mobile than subunit 1Bx7 differentiation between subunits 1By8 and 1By8* cannot be achieved using SDS-PAGE (uniform or gradient) (Marchylo *et al.*, 1992). Hence for reliable definitive identification of these alleles other methods, such as RP-HPLC, are often employed to facilitate HMW-GS identification.

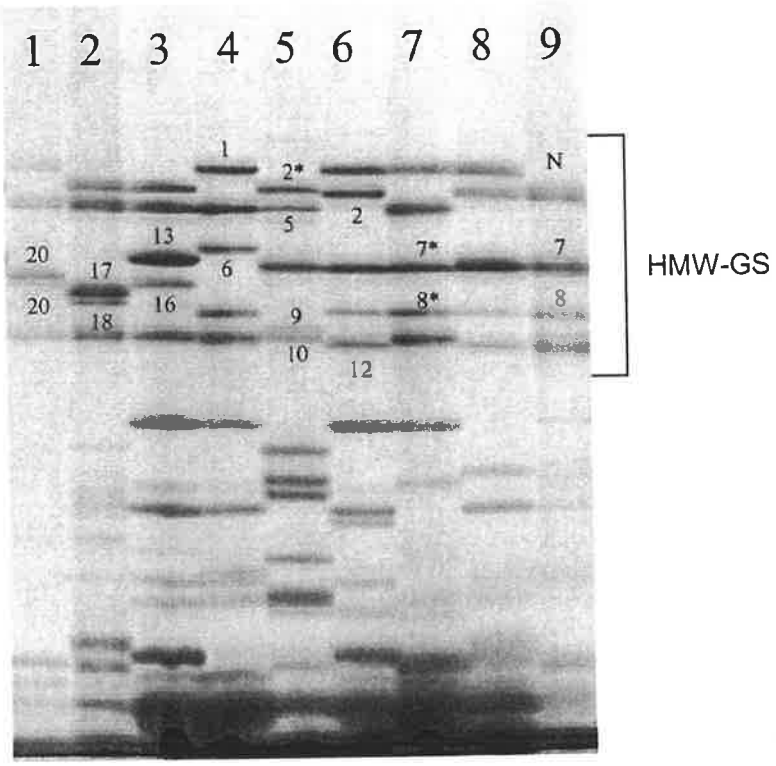


Figure 5-1 Uniform SDS-PAGE (8.5%) of HMW-GSs. Lane 1- Jabiru (1, 20, 5+10); 2- Wilgoyne (2*, 17+18, 5+10); 3- $WW80_{(3)}$ (2*, 13+16, 5+10); 4- Hope (1, 6+8*, 5+10); 5- Katepwa (2*, 7*+9, 5+10); 6- Janz (1, 7*+8, 2+12); 7- Norstar (1, 7*+8*, 5+10); 8- Sinvalocho (1, 7+8*, 2+12); and 9- Chinese Spring (N, 7+8, 2+12).

5.3.2 RP-HPLC

RP-HPLC separates proteins according to their surface hydrophobicities (Figure 5-2) and complements information provided by SDS-PAGE for different HMW-GSs (Gianibelli *et al.*, 2002). Hence, RP-HPLC is an important tool for identifying alleles of HMW-GSs at the *Glu-A1* locus (subunit 1Ax2* when *Glu-D1* subunits 2+12 are present, Figure 5-2, F), but particularly for differentiating between certain allelic variants at the *Glu-B1* locus, *Glu-B1b, u, al* and *ak* (Sutton, 1991; Marchylo *et al.*, 1992; Margiotta *et al.*, 1993; Lafiandra, 1994a; and Gianibelli *et al.*, 2002). This is because subunits 1By8 and 1By8* can only be distinguished by RP-HPLC. Subunit 1By8* is more hydrophilic than subunit 1By8 and as a consequence they have quite different elution times (Figure 5-2, A compared with B, C

and E). RP-HPLC also has the added advantage of being a suitable technique for quantifying HMW-GSs. Since subunits 1Bx7 and 1Bx7* have similar electrophoretic mobilities and surface hydrophobicities, discrimination between *Glu-B1* alleles containing either a 1Bx7 or a 1Bx7* is based on the quantitative measurement of these subunits, such as over-expression of subunit 1Bx7, relative to the total amount of HMW-GSs as well as which y-type subunit is also present (1By8 or 1By8*). The RP-HPLC chromatograms show the higher level of subunit 1Bx7 and the different elution time of subunit 1By8* detected in Sinvalocho (Figure 5-2, B) compared to the varieties Janz (Figure 5-2, A) and Norstar (Figure 5-2, C) which do not over-express subunit 1Bx7* (the *Glu-B1* subunit pair, 7+8, found in the cultivar Chinese Spring, is not shown).

This method of identifying subunits 1Bx7 and 1Bx7*, although helpful, is by no means definitive. Unequivocal identification the *Glu-B1al* allele has been reported using PCR (Cloutier *et al.*, 2000; Butow *et al.*, 2003; Juhasz et al., 2003a) which is especially useful in early generation, marker assisted selection. However, PCR does not determine levels of protein expression. This is important since some varieties that test positive for the *Glu-B1al* allele using PCR do not over-express subunit 1Bx7 (Gianibelli *et al.*, 2002), therefore confirmation of over-expression should still be obtained using RP-HPLC (Butow *et al.*, 2003).

In this study, HMW-GS composition was initially determined using uniform SDS-PAGE. In doing so, varieties that appeared to contain the *Glu-B1* subunits 1Bx7 or 1Bx7* and 1By8 or 1By8* were identified for further analysis using RP-HPLC. Varieties which possessed the *Glu-A1c* (null) allele were excluded to ensure that comparisons of the relative proportions of individual HMW-GSs were for varieties that contained five HMW-GSs. The nomenclature used was as described by Payne and Lawrence (1983) except for subunits 1Bx7* and 1By8* where that of Marchylo *et al.*, (1992) was adopted. An over-

expressing variety, Sinvalocho (Gianibelli *et al.*, 2002) and a normal expressing variety, Janz (Australian APW cultivar) were included in each run as positive and negative controls for over-expression, respectively. These varieties contain the same HMW-GS alleles, *Glu-A1a* and *Glu-D1a* but different *Glu-B1* HMW-GS, Sinvalocho having the *Glu-B1al* and Janz the *Glu-B1u* allele. They also served as reference samples to monitor the efficiency/column stability of the RP-HPLC method throughout this study. To investigate RP-HPLC elution and expression profiles of the more common HMW-GSs, varieties representative of other *Glu-B1* alleles were also included.

With regard to the RP-HPLC elution profiles of various reduced and alkylated HMW-GSs, the findings confirmed those that have been previously reported (Sutton, 1991; Marchylo *et al.*, 1989, 1992 and 1996; Margiotta *et al.*, 1993; Lafiandra *et al.*, 1994a, 1994b; Gianibelli *et al.*, 2002). As is evident in Figure 5-2, the *Glu-D1* y-type subunits are the first to elute with very similar elution times. Subunit 1Dy12 elutes at 19.79 ± 0.37 min compared with 19.8 ± 0.32 min for subunit 1Dy10. The next group of HMW-GSs to elute are the *Glu-B1* y-type subunits 1By20, 1By8*, 1By9 and 1By18, again with similar retention times of approximately 30.63 ± 0.23 min. These were followed by *Glu-D1* x-type subunits, 1Dx5 and 1Dx2, with elution times of 31.97 ± 0.32 min and 32.56 ± 0.33 min, respectively. At 33.28 ± 0.13 min the *Glu-B1* y-type subunit 1By16 elutes followed by subunit 1By8 at 35.05 ± 0.13 min. These two subunits were the only *Glu-B1* y-type HMW-GSs observed to elute after *Glu-D1* x-type subunits indicating that they are less hydrophilic than *Glu-D1* x-type and the other *Glu-B1* y-type subunits. *Glu-B1* x-type subunits elute next and in the following order : 1Bx13 (36.12 ± 0.2 min), 1Bx6 (38.33 ± 0.55 min), 1Bx7 (39.03 ± 0.39 min), 1Bx7* (39.54 ± 0.28 min), 1Bx17 (40.34 ± 0.09 min) and 1Bx20 (42.16 ± 0.22 min). The last HMW-GSs to elute are the x-type subunits encoded at the *Glu-A1* locus, 1Ax1 and 1Ax2*, again with similar retention times of 44.55

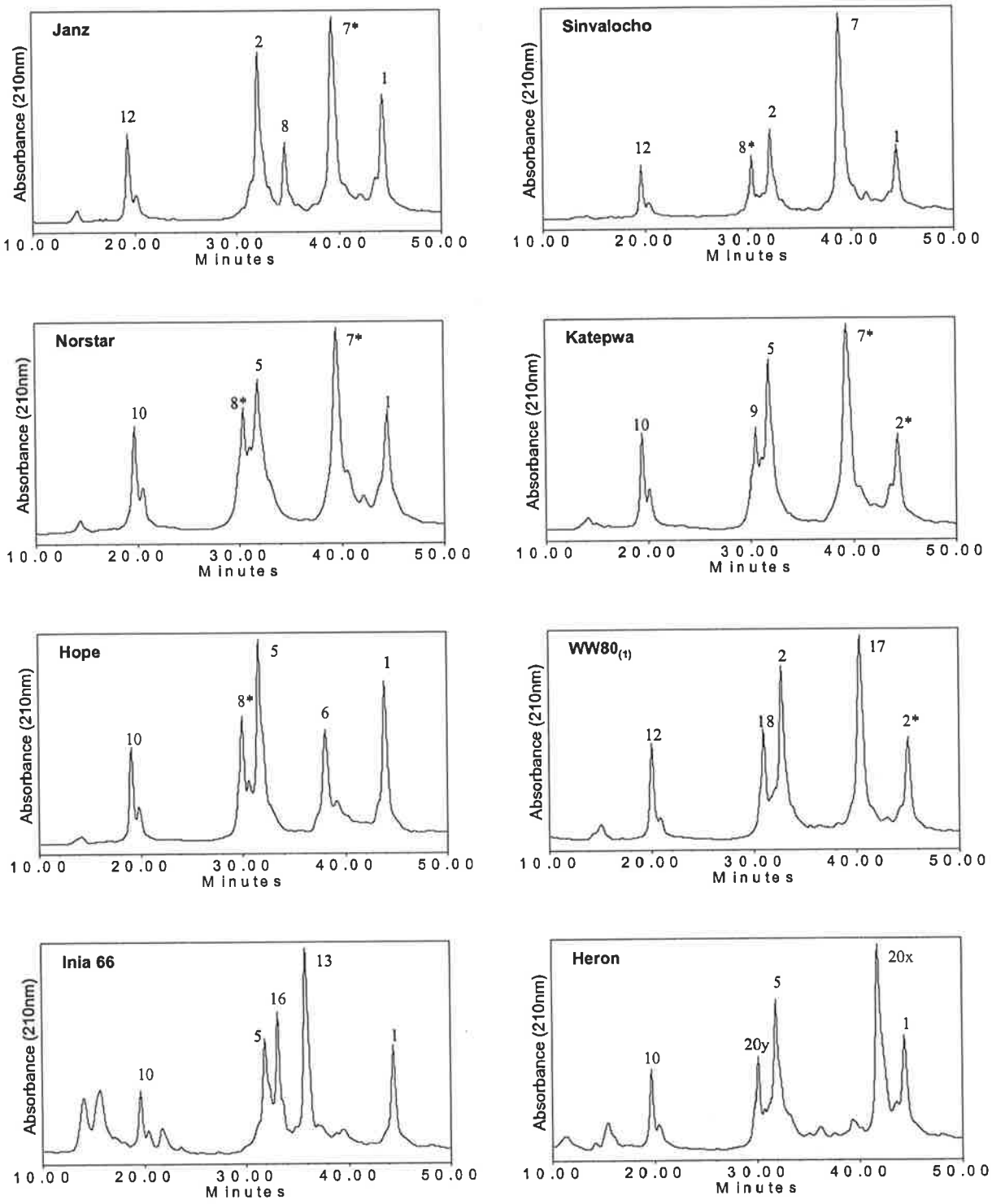


Figure 5-2 RP-HPLC chromatograms of various bread wheat cultivars differing in *Glu-B1* HMW-GS composition.

± 0.34 min and 44.71 ± 0.3 min, respectively. Inclusion of a single accession of *T. dicoccoides* (AABB) that expresses both 1Ax and 1Ay HMW-GSs revealed that the *Glu-A1* y-type subunit eluted just after the *Glu-D1* y-type subunits at approximately 21.43 min. This observation is consisted with those reported by Lafiandra *et al.* (1994a) and Margiotta *et al.* (1996). The order in which HMW-GSs elute is consistent, (1Dy; 1Ay; 1By; 1Dx; 1By16; 1By8; and 1Ax), but their retention times can vary. This variation can be attributed to differences in gradients produced by the RP-HPLC system but especially to minor differences in the concentration of tri-fluoro acetic acid (TFA) in the solvents used (data not shown). In addition, analysis of the varieties Barleta 7D (1, 7*+8*, 3+12) and Rudd (N, 6+8*, 3+12) revealed that RP-HPLC conducted as described here is unable to resolve 1Dx3 from 1By8* (data not shown). Clearly, due to the similarities in elution time observed for many of the *Glu-1* subunits, RP-HPLC alone is not a practical method for determining HMW-GS composition. However it is a powerful tool when used in conjunction with SDS-PAGE, particularly for its capacity to quantitate protein subunits more accurately than the densitometric analysis of stained gels (Kolster *et al.*, 1992; Lafiandra *et al.*, 1994b).

Peak area, calculated by intergrating the individual peaks and expressing the proportion of each peak relative to the sum of all peaks as a percentage, is indicative of the amount of subunit present (Table 5-1). Typical levels of expression for various HMW-GSs reported in this paper agree with the findings of Marchylo *et al.*, (1989) and can be summarised as follows : 1Bx > 1Dx > 1Ax > 1By ≈ 1Dy with the exception of *Glu-B1d* (6+8*) for which the average amounts of individual subunits were 1Dx (32.1 %) > 1Bx (19.9 %) > 1By (18.2 %) > 1Ax (16.6 %) > 1Dy (13.4 %). These results closely resemble those in Figure 5-1 (lane 4: Hope compared with lane 7: Norstar) and findings reported by Kolster *et al.* (1992) where the staining intensities of subunits 1Bx6 and 1By8* on SDS-PAGE appeared comparatively equal.

Cultivar	*Glu-B1*[a]			*Glu-A1*[a]		*Glu-D1*[a]				*Glu-B1*	*Glu-A1*	*Glu-D1*
		x	y	1x	2*x	2x	5x	12y	10y			
Sinvalocho [c]	7+8* [b]	47.6	10.3	13.7		19.4		9.0		57.9	13.7	28.4
Red River 68		44.5	7.6	16.5			20.9		10.5	52.1	16.5	31.4
Kukri		46.7	10.4	12.9			18.9		11.2	57.1	12.9	30.1
CD 87		44.6	10.0		15.6	20.8		9.0		54.6	15.6	29.8
Chara		42.4	12.0		13.4	22.2		10.1		54.4	13.4	32.3
Otane		51.9	11.0		12.4	18.0		6.8		62.9	12.4	24.8
Americano		46.4	12.1		12.7	19.3		9.5		58.5	12.7	28.8
Glenlea		46.2	10.7		12.4		22.5		8.2	56.9	12.4	30.7
Mean		**46.3**	**10.5**	**14.4**	**13.3**	**19.9**	**20.8**	**8.9**	**10.0**	**56.8**	**13.7**	**29.5**
SD		**2.79**	**1.40**	**1.89**	**1.35**	**1.61**	**1.80**	**1.25**	**1.57**	**3.25**	**1.54**	**2.30**
AUS 18049	17+18	30.4	14.4	16.2			24.2		14.8	44.8	16.2	39.0
Pavon F 76		35.6	12.7	18.7			21.3		11.6	48.3	18.7	32.9
WW-80		33.2	14.6		13.9	26.3		12.0		47.8	13.9	38.3
Mean		**33.1**	**13.9**	**17.5**	**13.9**	**26.3**	**22.8**	**12.0**	**13.2**	**47.0**	**16.3**	**36.7**
SD		**2.60**	**1.04**	**1.77**	**-**	**-**	**2.05**	**-**	**2.26**	**1.89**	**2.40**	**3.34**
Bacanora-T-88	7*+9	37.5	14.4		16.3		24.8		7.0	51.9	16.3	31.8
Thatcher		35.3	12.9		15.5		26.2		10.1	48.2	15.5	36.3
Katepwa		35.0	13.3		14.3		27.2		10.3	48.3	14.3	37.5
Sandy		31.6	11.5		18.7		27.0		11.1	43.1	18.7	38.1
Mean		**34.9**	**13.0**		**16.2**		**26.3**		**9.6**	**47.9**	**16.2**	**35.9**
SD		**2.44**	**1.20**		**1.86**		**1.09**		**1.80**	**3.62**	**1.86**	**2.85**
Norquay	13+16	30.3	15.7	17.6			23.8		12.6	46.0	17.6	36.4
Inia 66		29.7	19.3	22.5			21.4		7.1	49.0	22.5	28.5
Mean		**30.0**	**17.5**	**20.1**			**22.6**		**9.9**	**47.5**	**20.1**	**32.5**
SD		**0.42**	**2.55**	**3.46**			**1.70**		**3.89**	**2.12**	**3.46**	**5.59**
Janz [d]	7*+8 [b]	34.1	8.5	17.8		27.6		12.0		42.6	17.8	39.6
Frame		35.3	7.9	17.1		28.7		11.0		43.2	17.1	39.7
Pitic 62		33.7	9.7	14.8		29.4		12.4		43.4	14.8	41.8
Molineux		33.9	8.7	20.7			27.2		9.7	42.6	20.7	36.9
Era		36.4	10.4		13.9		28.9		10.4	46.8	13.9	39.3
Sunstar		34.7	8.3		20.4		26.5		10.2	43.0	20.4	36.7
Mean		**34.7**	**8.9**	**17.6**	**17.2**	**28.6**	**27.5**	**11.8**	**10.1**	**43.6**	**17.5**	**39.0**
SD		**1.02**	**0.94**	**2.43**	**4.60**	**0.91**	**1.23**	**0.72**	**0.36**	**1.60**	**2.80**	**1.92**
Barleta	7*+8*	32.9	13.5	16.9		26.6		10.2		46.4	16.9	36.8
Norstar		30.6	15.2	16.6			25.9		11.7	45.8	16.6	37.6
Mean		**31.8**	**14.4**	**16.8**		**26.6**	**25.9**	**10.2**	**11.7**	**46.1**	**16.8**	**37.2**
SD		**1.63**	**1.20**	**0.21**		**-**	**-**	**-**	**-**	**0.42**	**0.21**	**0.57**
Insignia	20+20	26.5	11.6	23.4			27.8		10.7	38.1	23.4	38.5
Olympic		27.3	11.3	22.7			27.2		11.5	38.6	22.7	38.7
Heron		30.6	11.9	21.2			27.6		8.7	42.5	21.2	36.3
Mean		**28.1**	**11.6**	**22.4**			**27.5**		**10.3**	**39.7**	**22.4**	**37.8**
SD		**2.17**	**0.30**	**1.12**			**0.31**		**1.44**	**2.41**	**1.12**	**1.33**
Hope	6+8*	20.7	16.3	19.1			32.8		11.2	37.0	19.1	44.0
Oroua		19.0	20.0		14.0		31.4		15.6	39.0	14.0	47.0
Mean		**19.9**	**18.2**	**19.1**	**14.0**		**32.1**		**13.4**	**38.0**	**16.6**	**45.5**
SD		**1.20**	**2.62**	**-**	**-**		**0.99**		**3.11**	**1.41**	**3.61**	**2.12**

[a] Means of duplicate analysis (% area);

[b] Subset of results;

[c] Means of positive control (n=20);

[d] Means of negative control (n=20);

Table 5-1 Quantitative analysis by RP-HPLC of the proportion (%) of various alkylated HMW-GSs relative to total HMW-GS area (calculated from chromatograms on the basis of relative area to total area and expressed as a percentage).

The B-genome (*Glu-B1* x + y) makes the greatest contribution to the percentage of HMW-GSs, compared with the A- and D-genomes (except in varieties with *Glu-B1e* (20x + 20y subunits) and *Glu-B1d* (6x+8*y subunits) where the contribution of the D genome is equal or slightly greater, respectively. Cultivars that possessed the *Glu-B1al* allele contained a significantly higher ($P<0.001$) proportion (56.8 ± 3.3 %) of HMW-GSs encoded by the B-genome than those that contained other *Glu-B1* alleles (Table 5-1). This increase in the proportion of *Glu-B1* subunits can be attributed to the over-expression of subunit 1Bx7 which is accompanied by a proportional reduction of HMW-GSs encoded by the A- and D-genomes. Since *Glu-B1al* is associated with a higher R_{max} and Ext. than other *Glu-B1* alleles it is evident that the proportion of *Glu-B1* subunits relative to the total amount of HMW-GSs expressed, has a major effect on dough strength. Clear differentiation between *Glu-B1* alleles was observed when the relative proportions of the 1Bx subunits were compared to total x-type HMW-GS ratio, the ratio of 1Bx subunits to 1Dx subunits and the x- to y-type HMW-GS ratio (Table 5-2).

The mean ratio of *Glu-B1* x-type subunits to total x-type HMW-GSs in cultivars which possess the *Glu-B1al* allele (0.58) is significantly higher ($P<0.001$) than for those having other *Glu-B1* alleles. Statistical analysis of cultivars with the *Glu-B1* alleles *i* (0.45), *f* (0.45), *c* (0.41), *u* (0.43) and *ak* (0.42) showed that the Bx:Ax+Bx+Dx ratio was not significantly different ($P<0.05$) in this group, while it was significantly higher ($P<0.05$) than in cultivars that possess the *Glu-B1e* (0.36) and *Glu-B1d* (0.29) alleles. *Glu-B1e* and *Glu-B1d* were not significantly from each other (Table 5-2). This division of *Glu-B1* alleles into three sub-groups (Group 1: *Glu-B1al*; Group 2: *Glu-B1i*, *f*, *c*, *u* and *ak*; and Group 3: *Glu-B1e* and *d*) reflects their known influence on dough strength, which is extra strong, strong-to-medium and weak, respectively. Further evidence that supports the division of *Glu-B1* alleles into subgroups and highlights their importance in determining R_{max} is apparent when the *Glu-B1* alleles are ordered (descending) according to the

Glu-B1 allele		Bx:Ax+Bx+Dx[a]	Bx:Dx	x:y
al (7+8*)	Mean	0.58 **a**	2.31	4.08
	SD	0.03	0.31	0.43
i (17+18)	Mean	0.45 **b**	1.40	2.77
	SD	0.02	0.24	0.34
c (7*+9)	Mean	0.45 **b**	1.33	3.42
	SD	0.03	0.14	0.18
f (13+16)	Mean	0.41 **b**	1.33	2.66
	SD	0.01	0.08	0.18
u (7*+8)	Mean	0.43 **b**	1.24	4.06
	SD	0.02	0.05	0.38
ak (7*+8*)	Mean	0.42 **b**	1.21	2.97
	SD	0.01	0.04	0.36
e (20+20)	Mean	0.36 **c**	1.02	3.57
	SD	0.02	0.08	0.25
d (6+8*)	Mean	0.29 **c**	0.62	2.22
	SD	0.01	0.02	0.59

[a] Values followed by the same letter are n.s. (P<0.05)

Table 5-2 Comparison between the relative proportions of 1Bx subunits to total x-type HMW-GSs, the ratio of 1Bx to 1Dx subunits and the x- to y-type subunit ratio for various *Glu-B1* alleles.

proportion of 1Bx subunits relative to 1Dx subunits (Table 5-2). The order in which the *Glu-B1* alleles rank (*Glu-B1* : $al > i = c = f = u = ak > e > d$) closely resembles predictions for R_{max} ($al > f = i = u = c > e$) obtained using "Glutenin Quality Prediction" software (Eagles *et al.*, 2004; Eagles, pers. comm.).

The over-expression of the x-type subunit 1Bx7, which leads to an increase in proportion of x-type subunits as indicated by the x:y ratio (Table 5-2), may be the mechanism by which the *Glu-B1al* allele exerts such a large positive effect on dough strength. Although reconstitution studies have shown that synergistic effects exist between both x- and y-type HMW-GSs since the presence of both types of subunits positively influence mixing properties (Bekes *et al.*, 1994) the contribution of x- and y- type HMW-GSs to gluten

strength is very different (Seilmeier *et al.*, 1991, cited by Wieser *et al.*, 1994). Seilmeier *et al.* (1991) found that the amount of x-type subunits correlated highly with SDS-sedimentation volume (r^2 = +0.68) and R_{max} (r^2 = +0.89), whereas y-type subunits did so only weakly (r^2 = +0.17 and +0.29, respectively). Except for *Glu-B1u* (4.06 ± 0.38), the *Glu-B1al* allele corresponded with an increase in the x:y ratio (4.08 ± 0.43) compared with a mean 2.94 ± 0.32 for the other *Glu-B1* alleles (Table 5-2). The unexpected high x:y ratio observed for *Glu-B1u* was not the result of increased x-type subunits but a reduction in y-type HMW-GSs (Table 5-1). As reported by Lafiandra *et al.* (1994b), subunit 1By8 was produced in smaller quantities (8.92 ± 0.94 %) than the other 1By subunits (14.29 ± 2.59 %).

Another possible explanation for the increase in dough strength associated with the *Glu-B1al* allele is that it may be the result of an increase in the quantity of total HMW-GSs relative to total storage protein (Marchylo *et al.*, 1989; Sutton, 1991). This and/or structural differences in the relevant HMW-GSs (Marchylo *et al.*, 1992) possibly lead to an increase in the size of glutenin polymers present in the gluten. Percent SDS-unextractable polymeric protein (%UPP), as determined by SE-HPLC, is a parameter indicative of polymer size which has been shown to correlate highly (r^2 = +0.89) with R_{max} (Gupta *et al.*, 1993; Ciaffi *et al.*, 1996a; MacRitchie, 1999; Gianibelli *et al.*, 2002). Evidence for the increase in polymer size has recently been reported where varieties that contain the *Glu-B1al* allele were found to have a higher %UPP than non over-expressing varieties (Butow *et al.*, 2002; Juhasz *et al.*, 2003b).

The increase in the proportion of subunit 1Bx7 in varieties which contained the *Glu-B1al* allele was not at the expense of another single HMW-GS. The relative amounts of the other subunits were proportionately decreased so that the level of expression of individual subunits ranked in the same order as for varieties where over-expression was not observed,

1Bx > 1Dx > 1Ax > 1By ≈ 1Dy (Figure 5-2). The proportion (% area) of subunit 1Bx7* present in normal expressing varieties was 34.7 ± 1.88% and significantly lower (P<0.001) than that of 1Bx7 in over-expressing varieties (46.5 ± 2.5%) (Table 5-3). The difference (46.5 to 34.7) in values reported here are greater than those previously reported (Marchylo *et al.*, 1992; Gianibelli *et al.*, 2002) and most likely due to differences in protein extraction procedures. To precipitate all the HMW-GSs, it was necessary to conduct this step at 4 °C. Also, after commencement of this work, it was found that even following two extractions, approximately 10 % of the HMW-GSs still remained in the flour residue (data not shown). Protein extraction for RP-HPLC analyses could be improved by conducting a third extraction step.

5.3.3 Origin of the Glu-B1al allele

Differences in the electrophoretic mobility of subunits 1Bx7 and 1Bx7* was first described by Payne *et al.* (1981). In 1991, Sutton observed that subunit 1Bx7 was over-expressed in the New Zealand cultivar, Otane, which has a high R_{max}, and suggested that the increase in the proportion of subunit 1Bx7 could be a determining factor. Soon after, Marchylo *et al.* (1992), reported that the over-expression of subunit 1Bx7 was always accompanied by subunit 1By8* (designated *Glu-B1al*) and that this allele was present in a number varieties, including Glenlea. Glenlea, a specialty Canada Western Utility variety with extra strong dough properties was released in the 1970's. In 1993 a new market class, Canada Western Extra Strong (CWES), based on the variety Glenlea, was established to reflect the demand for wheats that produce extra strong doughs. CWES is ideal for blending purposes, the production of frozen dough products that maintain baking quality after thawing as well as for bread produced using the 'sponge and dough' method (Cereal Research Centre, Agriculture and Agri-Food Canada, 2003). Further, the findings of Butow *et al.* (2002) indicated that extra-strong cultivars may also be suited to the production of salt-reduced bread products.

Cultivar	*Glu-1*[a]			Relative Proportion of subunit 7 to total HMW-GS (% area)[b]
	1A	*1B*	*1D*	
Americano	2*	7+8*	2+12	46.4
Bajio	2*	7+8*	5+10	50.6
Biggar	1	7+8*	2+12	44.7
Bluesky	2*	7+8*	5+10	46.4
Calidad[e]	2*	7+8*	5+10	47.5
CD87	2*	7+8*	2+12	44.6
Chara	2*	7+8*	2+12	42.4
Emu S[e]	2*	7+8*	2+12	48.2
Endeavour	2*	7+8*	2+12	51.7
Gallo	2*	7+8*	5+10	49.3
Glenlea	2*	7+8*	5+10	46.2
Glenman	1	7+8*	5+10	43.2
Grajo S	1	7+8*	5+10	47.0
Jaral 66	1	7+8*	2+12	47.6
Kohika	2*	7+8*	2+12	52.0
Klein Sin Rival	2*	7+8*	2+12	45.9
Klein Universal II	2*	7+8*	2+12	44.1
Kukri	1	7+8*	5+10	46.7
Laura	1	7+8*	5+10	46.1
Oslo	1	7+8*	2+12	45.8
Otane	2*	7+8*	2+12	51.9
Prospur	1	7+8*	5+10	43.9
Red River 68	1	7+8*	5+10	44.5
Roblin	2*	7+8*	5+10	42.8
Sinvalocho[c]	2*	7+8*	2+12	47.6
TZPP[g]	2*	7+8*	5+10	43.8
Tobari 66	1	7+8*	5+10	46.9
Tobari 66 S	1	7+8*	5+10	45.4
Universal II	2*	7+8*	2+12	44.2
Wheaton	2*	7+8*	5+10	46.1
Wildcat	2*	7+8*	5+10	46.3
Woodpecker S	2*	7+8*	2+12	45.9
				46.4 +/- 2.5

Cultivar	*Glu-1*[a]			Relative Proportion of subunit 7* to total HMW-GS (% area)[b]
	1A	*1B*	*1D*	
Banks	2*	7*+8	2+12	38.5
Barunga	1	7*+8	5+10	31.5
Batavia	1	7*+8	2+12	36.1
Chino	2*	7*+8	2+12	35.5
Cook	1	7*+8	2+12	36.3
Cunningham	1	7*+8	2+12	32.8
Era	2*	7*+8	5+10	36.4
Frame	1	7*+8	5+10	35.3
Hira	1	7*+8	5+10	34.2
Janz[d]	1	7*+8	2+12	34.1
Molineux	1	7*+8	5+10	33.9
Perouse	2*	7*+8	2+12	34.3
Pitic 62	1	7*+8	2+12	33.7
Pondera	1	7*+8	5+10	33.5
RAC 750	2*	7*+8	2+12	35.1
Sunstar	2*	7*+8	5+10	34.7
Bacanora 88	2*	7*+9	5+10	34.4
Bacanora T 88	2*	7*+9	5+10	37.5
Katepwa	2*	7*+9	5+10	35.0
Sandy	2*	7*+9	5+10	31.6
Thatcher	2*	7*+9	5+10	35.3
Barleta[f]	1	7*+8*	2+12	35.3
Norstar	1	7*+8*	5+10	30.6
				34.6 +/- 1.85

[a] Nomenclature of Payne and Lawrence (1983) except for subunit 7* and 8* as described by Marchylo *et al*. (1992).
[b] Means of duplicate analysis
[c] Means of positive control (n=20)
[d] Means of negative control (n=20)
[e] Means of duplicate analysis for two accessions
[f] Means of duplicate analysis for three accessions
[g] Tezanos Pintos Precoz
[h] Mean +/- standard deviation

Table 5-3 Quantitative analysis using RP-HPLC for the expression of alkylated *Glu-B1* HMW-GSs 1Bx7 and 1Bx7* in bread wheat cultivars.

The reason for the over-expression of subunit 1Bx7 has been attributed to either a duplication of the 1Bx7 gene where both gene copies are functional (Lukow *et al.*, 2002; D'Ovidio *et al.*, 1997) and/or an increased efficiency in the transcription and/or translation of the 1Bx7 gene, as a difference in the promoter region (a 43-nucleotide insert) of this gene has been identified (Lukow *et al.*, 2002; Cloutier *et al.*, 2000).

One of the goals of this work was to identify named varieties, of different origin that contain the *Glu-B1al* allele and over-express subunit 1Bx7. These results are presented in Table 5-3 and Table 5-4. Many of the over-expressing varieties identified in this paper have been previously reported, namely, Otane (Sutton, 1991), Glenlea, Laura, Biggar, Oslo, and Roblin (Marchylo *et al.*, 1992), Red River 68 (D'Ovidio *et al.*, 1997), Kukri and Chara (Butow *et al.*, 2002) and Sinvalocho (Gianibelli *et al.*, 2002). Pedigrees of these varieties using dendrograms produced by the International Wheat Information System (IWIS) (Payne *et al.*, 2000) were used identify common ancestors. A common ancestor, the Argentinian variety Tezanos Pintos Precoz (TZPP), was identified as the over-expressing parent for wheat cultivars from Mexico (Jaral 66, Tobari 66 and Bajo), Canada (Glenlea) and the USA (Red River 68, Prospur and Glenman) all of which carry the *Glu-B1al* allele. The origin of the *Glu-B1al* allele could be traced back further so that the earliest named variety (1922) which contained the *Glu-B1al* allele could be identified. This was the Argentinian wheat cultivar, Klein Universal II and its sister line, Universal II. It is also likely that Americano 44D (accession unavailable), another sister line of Klein Universal II, possesses this allele since an over-expressing variety called Americano has been released in Chile.

Also of note is the presence of the *Glu-B1al* allele in the Australian breeding line, CD87. Based on its generally accepted pedigree (Pavon F76 x Condor, IWIS) the origin of *Glu-B1al* could not be established since neither Pavon F76 or Condor over-express the 1Bx7

Origin	Cultivar	Accession	Origin	Cultivar	Accession
Argentina	Calidad (x2)	AUS 12040	***Mexico***	Bajio	AUS 28
		AUS 15132		Emu S (x2)	AUS 17224
	Klein Sin Rival	AUS 7486			AUS 20402
	Klein Universal II	AUS 2774		Flycatcher S	AUS 22650
	Sinvalocho	AUS 4339		Gallo	AUS 15933
	Tezanos Pintos Precoz	AUS 15037		Grajo S	AUS 17229
	Universal II	AUS 7459		Jaral 66	AUS 368
				Tobari 66	AUS 1395
Australia	CD87	AUS 91169		Tobari 66 S	AUS 16015
	Chara	AUS 30031		Woodpecker S	AUS 19506
	Kukri	AUS 29472			
			New Zealand	Endeavour	
				Kohika	
Canada	Biggar			Otane	AUS 30426
	Bluesky				
	Glenlea	AUS 15566	***United States***	Glenman	AUS 25110
	Laura			Oslo	
	Roblin	AUS 24970		Prospur	AUS 18641
	Wildcat			Red River 68	AUS 10947
				Wheaton	AUS 22906
Chile	Americano	AUS 7245			

Table 5-4 Geographical origin of cultivars that contain the *Glu-B1al* allele and exhibit over-expression of the HMW-GS 1Bx7.

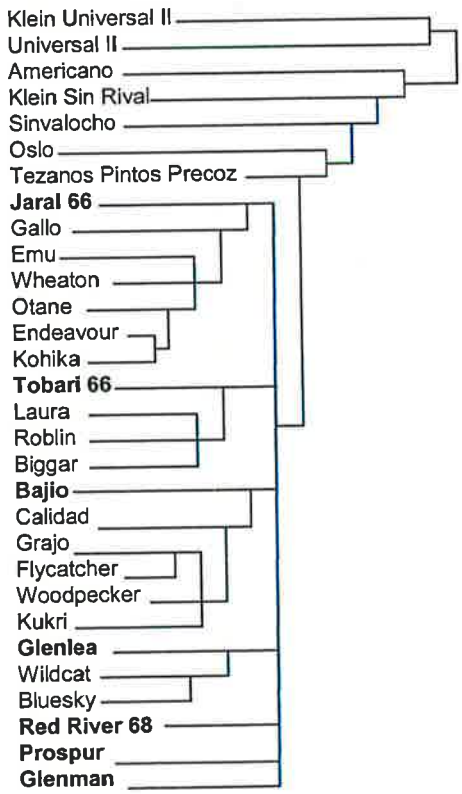


Figure 5-3 Genetic relationship between the over-expression of 1Bx7 in named cultivars of different origin and its source in Argentinian bread wheats. Dendogram produced from information obtained using IWIS, CIMMYT.

subunit. However, re-examination of old breeding records has revealed that the pedigree of CD87 is more likely to be Bluebird/Tobari66//CianoF67/Huacamayo/3/Condor (Eagles, pers. comm.). From this it can be concluded that the presence of the *Glu-B1al* allele in CD87 and the Australian cultivar, Chara (CD87 progeny) originated from the Mexican variety, Tobari 66. The genealogical relationships between over-expressing varieties in this study and Klein Universal II, via at least one parent, are summarised in Figure 5-3.

5.4 CONCLUSION

The capacity to produce good bread appears to have accompanied the introduction of the D genome into tetraploid wheat resulting in the evolution of hexaploid wheat (Simmonds, 1989). For many years the superiority of *Glu-D1d* (5+10) compared to *Glu-D1a* (2+12) in relation to bread making quality has been realised (Payne *et al.*, 1987). This phenomenon has received considerable research effort and the positive effects of the subunit pair 5+10 have been attributed to 1Dx5 subunit which contains an extra cysteine (Mackie *et al.*, 1996b).

However, a high proportion of 1Bx subunits compared to 1Dx subunits (≈ 2.3:1, *Glu-B1al*) correlated with varieties reported to have extra strong dough properties (Butow *et al.*, 2002), while a 1Bx:1Dx ratio of ≈ 1.3:1 (for *Glu-B1* alleles : *i*, *f*, *c*, *u* and *ak*) was typical of varieties with good dough strength characteristics. In varieties which contain *Glu-B1* alleles reported to produce weak doughs the 1Bx:1Dx value was ≈ 1:1 (*Glu-B1e*) and ≈ 0.6:1 (*Glu-B1d*). This suggests that the proportion of 1Bx to 1Dx subunits has a major influence on dough strength. The importance of the B genome in relation to dough strength in bread wheat has been reported (Metakovsky *et al.*, 1990) and is supported by evidence that some durum wheats (*Triticum durum*, AABB) also have the ability to produce good loaves of bread (Lagudah *et al.*, 1987; Singh *et al.*, 1998). However, this

work pertaining to the *Glu-B1al* allele demonstrates just how underestimated the importance of the B genome has been. It should also be noted, based on the findings reported in Chapter 4, the positive effects of *Glu-B1al* and *Glu-D1d* on dough strength would most likely be additive and may result in doughs that are over-strong. A possible solution would be to substitute the less extensible *Glu-D1d* (5+10) allele with *Glu-D1a* (2+12) and/or ensure alleles that convey high extensibility, such as *Glu-B3i*, are encoded at the other glutenin loci. Further work, preferably with isogenic lines is necessary to ascertain unequivocally the relationship between the over-expression of subunit 1Bx7 and dough rheology.

Finally, the rarity of the *Glu-B1al* allele in Australian germplasm contrasts with the high frequency found in Argentinian wheat cultivars. Australian wheat breeding programs have the opportunity to significantly improve bread making quality by the positive selection of the *Glu-B1al* allele. Already, the recognized high quality dough characteristics of CD87 has resulted in it being widely used as a parent for the development of new germplasm in Victorian breeding programs.

CHAPTER 6

DEVELOPMENT AND VALIDATION OF A SIMPLE TURBIDITY ASSAY FOR PREDICTING DOUGH STRENGTH

6.1 INTRODUCTION

Glutenin, the main protein component responsible for the difference in end-use quality among different wheat genotypes, can vary in molecular weight distribution arising from the capacity of its subunits to cross-link via disulfide bonds. Investigations into the relationship between protein composition and functionality has revealed that dough strength is mainly related to the proportion of polymeric protein that is above a critical size range since it is this protein fraction that is able to participate in effective entanglements (MacRitchie *et al.*, 1992). There are two types of polymeric protein, those that are readily extractable and those that are too large and require sonication for extraction into neutral detergent solution. While the actual molecular weight distribution of native glutenin is difficult to measure, the amount of 'functional glutenin' or large, unextractable polymeric protein (%UPP) can be measured using SE-HPLC. Whilst this method represents a valuable research tool, it is too slow and costly for routine screening in a wheat breeding program. This chapter describes the successful development and validation of an alternate, simpler method based on turbidity for estimating %UPP and hence predicting dough strength. Application of the Turbidity assay and how it facilitated the selection of *T. dicoccoides* accessions with a high %UPP is presented in Chapter 7 of this thesis.

6.2 MATERIALS AND METHODS

A subset of named varieties and breeding lines that were part of AGT's 2001 callibration set (Table 3-2, Chapter 3) were used to develop and validate the Turbidity assay. NIR, farinograph and extensograph quality data were provided by AGT. The methods employed

by AGT to obtain the quality data are described in Chapter 3. %UPP was determined using SE-HPLC as outlined in Chapter 3.

6.3 RESULTS AND DISCUSSION

6.3.1 Development of a Turbidity assay for percent SDS-insoluble protein

The same protein extraction method as described for SE-HPLC in materials and methods (Chapter 3) was employed but with some minor modifications aimed at increasing throughput. Flour extracts (36 samples per assay) were weighed into a 96 deep-well block (2 mL) instead of 1.5 mL Eppendorf tubes (Figure 6-1). To each well 1 mL of PEB was added and the samples gently mixed with a paperclip to ensure the samples were evenly wetted. The block was sealed and placed on a Thermolyne shaker (Type 50000 Maxi-Mix III, Sybron) for 2 min at ≈ 1500 rpm. After a 10 min centrifugation (1,968 x g, Sigma 3-10), 20 µL aliquots of the supernatant (SDS-soluble fraction) were transferred to rows B, C and D of two separate 96 well micro-titre plates (Figure 6-1). The remaining supernatant was then decanted and the block placed face down on absorbent paper for 5 min.

To obtain the SDS-insoluble protein fraction, 1 mL of PEB was added to the pellets that remained in the block. Samples were then sonicated for 30 sec in the block as per the SE-HPLC protocol (Chapter 3, pg 70). After the samples were again centrifuged for 10 min (1,968 x g, Sigma 3-10), 20 µL aliquots of the supernatant were then transferred to rows F, G and H of the same two 96 well micro-titre plates, as shown in Figure 6-1. To rows A and E, blank samples were prepared by adding 20 µL of PEB.

To precipitate total protein present in the SDS-soluble and SDS-insoluble fractions, 150 µL of 10% trichloroacetic acid (TCA) was added and the turbidity was left to develop at room temperature for 10 min. The amount of protein present in each sample was determined

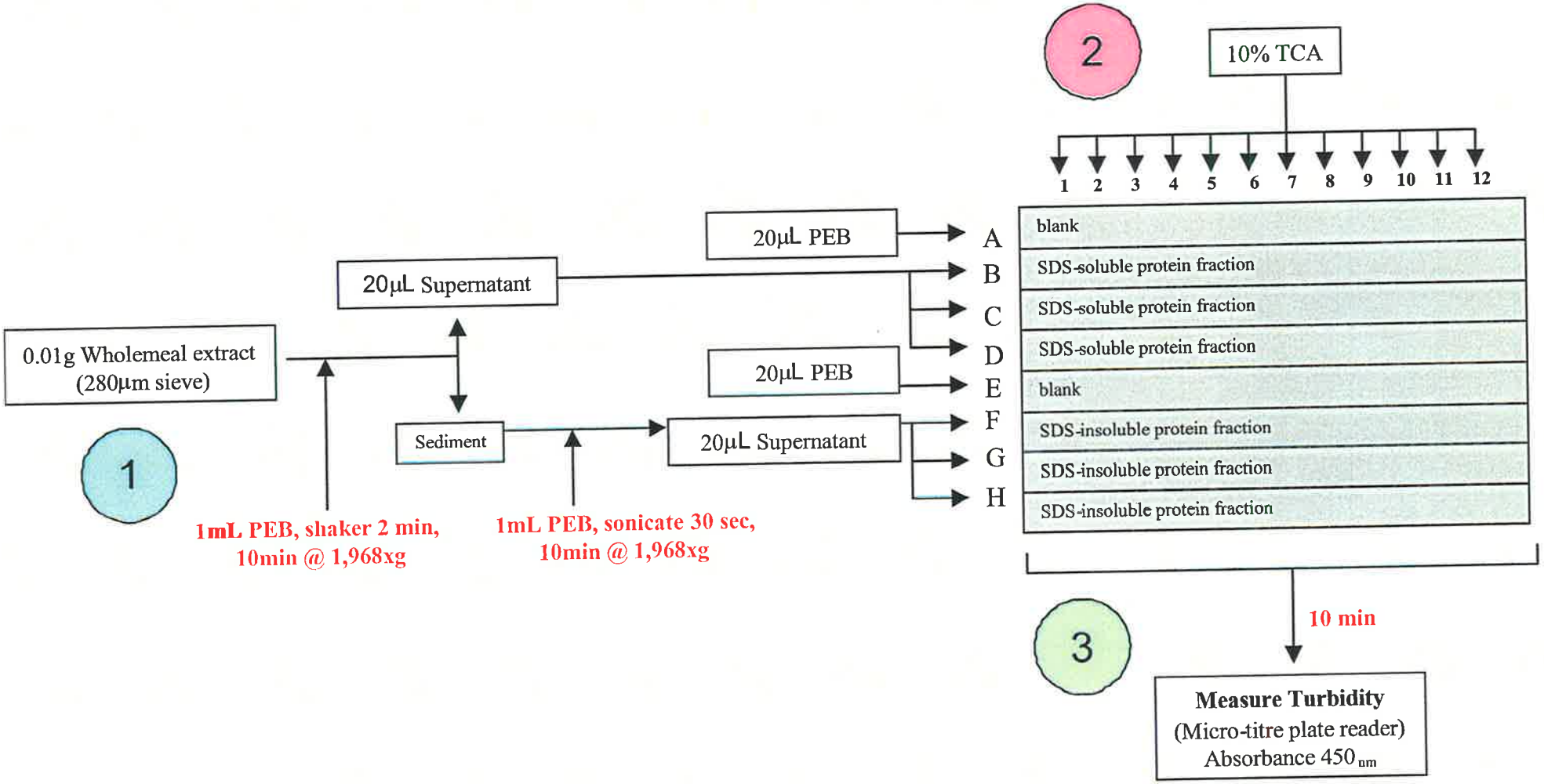


Figure 6-1 Diagrammatic representation of the protocol used to conduct the Turbidity assay. **Step 1**. Extract protein fractions and aliquot into micro-titre plate (in duplicate). **Step 2**. Conduct precipitation step. **Step 3**, Measure absorbance at 450_{nm}

spectrophotometrically at 450nm (Choi *et al.*, 1993; Wieser, 2000) using a Titertek Plus (MS2 Reader, ICN Flow®) micro-titre plate reader. %IP was calculated by dividing the absorbance of the SDS-insoluble protein fraction by the sum of the absorbance of the SDS-soluble and SDS-insoluble protein fractions and multiplying this figure by 100.

6.3.2 Validation of the Turbidity assay

The positive correlation observed for the SE-HPLC parameters (Table 6-1), %UP and %UPP ($r = 0.97$, $P \leq 0.001$) suggests that a high percentage of SDS-unextractable polymeric protein coincides with a high percentage of total SDS-unextractable protein (%IP) present in flour. This is the principle on which the potential usefulness of developing a Turbidity assay was based. %IP as determined by the Turbidity assay is a simple relative measure of the molecular weight distribution (MWD) of protein based on the well known inverse relationship between solubility and molecular size. As shown in Table 6-1 and Figure 6-2, %IP is positively and highly correlated with %UPP measured on SE-HPLC ($r = 0.92$, $P \leq 0.001$).

The relationships between %IP and other rheological parameters derived from larger scale physical dough tests such as farinograph dough development time (FDDT) and dough stability (FDS) and extensograph maximum resistance (R_{max}) and dough strength (Area) were also highly significant and remarkably similar to the correlations obtained using %UPP (Table 6-1). Further, the correlation coefficients reported here are based on linear regressions but higher r^2 values were obtained using a logarithmic regression between SE-HPLC (%UPP) and farinograph (FDDT and FDS) and extensograph (R_{max} and Area). Similarly, logarithmic regressions between turbidity (%IP) and these farinograph and extensograph parameters were also higher than they were for linear regression. Examples of this can be seen in Figure 6-3 and Figure 6-4 which show both the linear and logarithmic trendlines for R_{max} x %UPP and R_{max} x %IP regressions, respectively.

Attribute [a]	NIR [b]		Farinograph [b]				Extensograph [b]			SE-HPLC [b]				
	FP	PSI	FWA	FDDT	FDS	FDBD	Ext.	R_{max}	Area	TP	%UP	%PPP	%FPP	%UPP
%UPP	0.45 ***	-0.46 ***	0.26	0.68 ***	0.87 ***	-0.90 ***	0.47 ***	0.89 ***	0.87 ***	0.49 ***	0.97 ***	0.44 **	0.57 ***	-
%IP	0.43 **	-0.46 ***	0.28 *	0.68 ***	0.81 ***	-0.85 ***	0.49 ***	0.81 ***	0.81 ***	0.51 ***	0.92 ***	0.49 ***	0.58 ***	0.92 ***

[a] FP = flour protein (%); PSI = grain hardness (≥ 24 = soft); FWA = water absorption (%); FDDT = dough development time (min); FDS = dough stability (min); FDBD = dough breakdown (BU); Ext. = extensibility (cm); R_{max} = maximum resistance (BU); Area = dough strength (cm^2); TP = total protein (Area x 10^{-5}); %IP = percentage of SDS-insoluble protein in total protein; %PPP = percentage of flour polymeric protein in total protein; %FPP percentage of polymeric protein in flour; %UPP = percentage of SDS-unextractable polymeric protein in total polymeric protein; %IP = percentage of SDS-insoluble protein relative to total protein.

[b] *, **, *** = significantly correlated at P≤0.05, P≤0.01 and P≤0.001% probability, respectively.

Table 6-1 Correlation coefficients of linear regression between %UPP and %IP and quality parameters of Buhler milled flours derived from 52 hexaploid varieties grown at the University of Adelaide, Roseworthy Campus, SA in 2001.

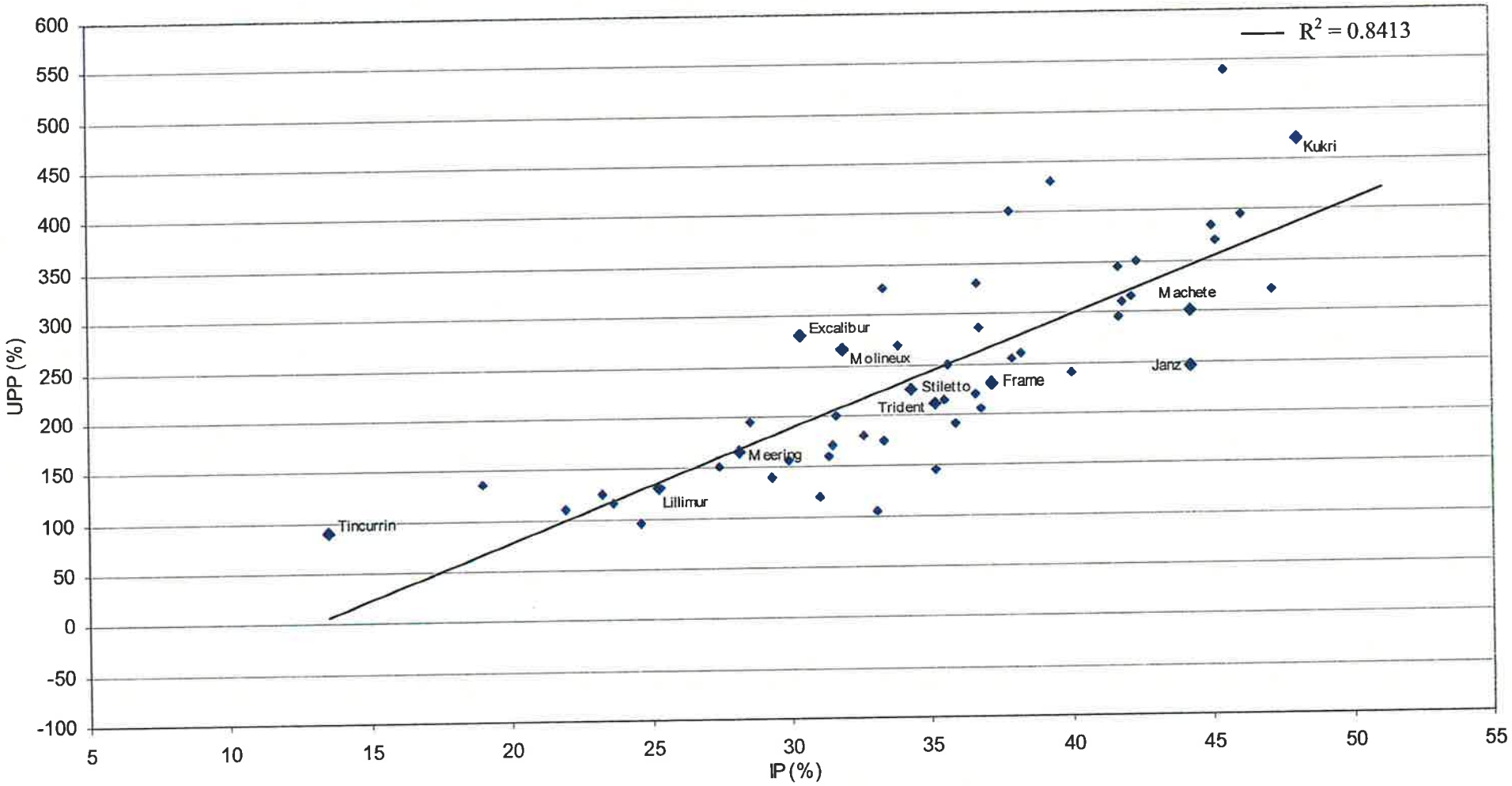


Figure 6-2 Linear regression analysis between the percentage of SDS-insoluble protein in total protein (%IP; Turbidity assay) and the percentage of SDS-unextractable polymeric protein in total polymeric protein in flour (%UPP; SE-HPLC). Correlation coefficient r = 0.92, P ≤0.001.

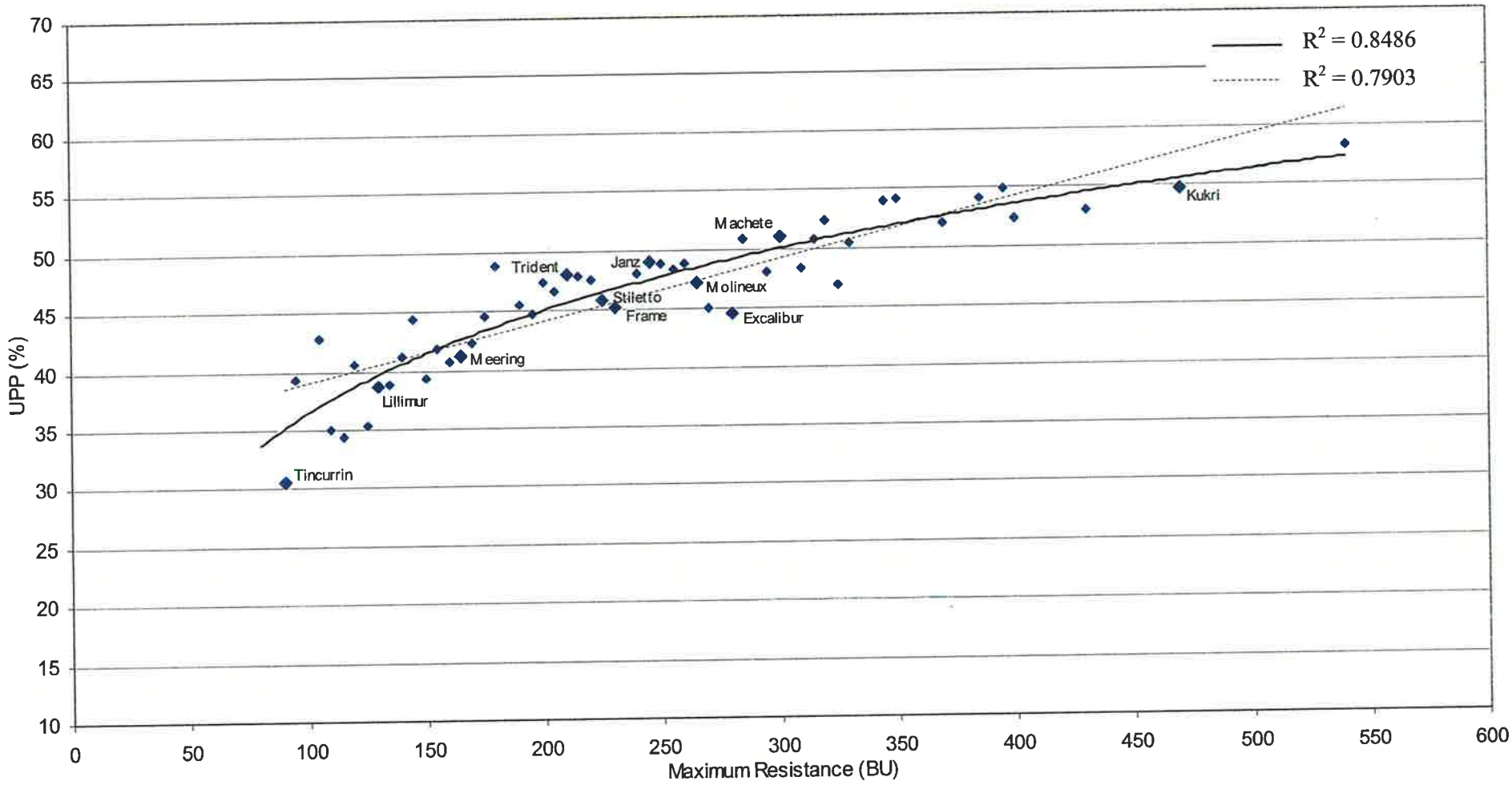


Figure 6-3 Linear and logarithmic regression analysis between the extensograph parameter, maximum resistance (R_{max}) and the percentage of SDS-unextractable polymeric protein in total polymeric protein (%UPP) as determined by SE-HPLC. Linear correlation coefficient $r = 0.89$, $P \leq 0.001$.

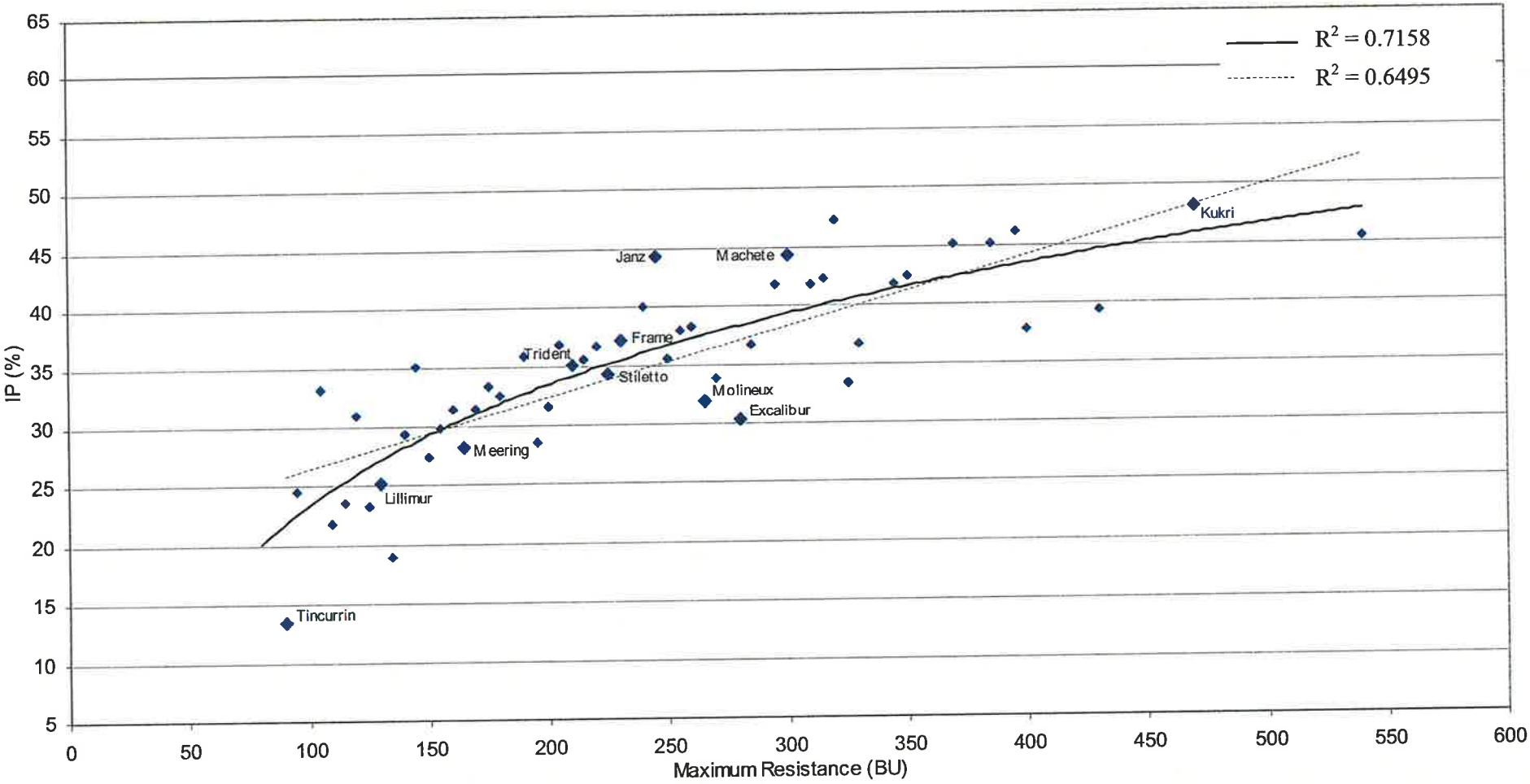


Figure 6-4 Linear and logarithmic regression analysis between the extensograph parameter, maximum resistance (R_{max}) and the percentage of SDS-insoluble protein relative to total protein (%IP) as determined by the Turbidity assay. Linear correlation coefficient r = 0.81, P ≤0.001.

The rate limiting step in the Turbidity assay is the sonication of the insoluble fraction. As an alternative to sonication, 1% w/v DTT (Wieser, 2000) was evaluated for its capacity to efficiently solubilise the insoluble fraction. Similarly, 80% acetone and 83% propan-2-ol were compared with TCA in the protein precipitation step (Melas *et al.*, 1994; Hou and Ng, 1995; Wieser, 2000). None of these alternatives were as robust and, in particular, all appeared to be susceptible to temperature fluctuations, a factor previously noted by Wieser (2000).

The Turbidity assay reported here has proved to be robust and has provided consistent results. Repeatability was more influenced by the extraction procedure than by the precipitation and subsequent turbidimetic measurement. This could be due to 10% TCA being more efficient at precipitating protein than 80% acetone or 83% propan-2-ol and/or because the sonicated protein present in the insoluble fraction has a greater MWD. Controlled sonication enables solubilisation of the largest glutenin molecules by breaking them down with mechanical sheer, but as polymer theory predicts, the degradation products still fall within the size range of soluble polymeric protein (MacRitchie, 1999). Consequently, the protein in the insoluble fraction remains in larger molecules than when it is chemically reduced into protein subunits. If the reliability of the Turbidity assay for predicting SE-HPLC %UPP is governed by the MWD of the proteins present in the insoluble fraction, it is not surprising that protein reduction to subunits is not a suitable alternative to sonication.

Reduction followed by reoxidation prior to precipitation is another option that is currently being investigated as an alternative to sonication. Buonocore *et al.* (1996b) showed that when reoxidised using potassium bromate *in vitro*, protein peptides formed cross-linked polymers, the size of which increased with the number of cysteine residues. Replacement of sonication with reduction/oxidation would reduce the time/labour involved in

conducting the Turbidity assay and enable the number of samples analysed per day to be increased. Currently, the Turbidity assay allows for the analysis of 36 samples (2 controls plus 34 unknowns) per assay and it is possible to conduct 2 assays/day. Thus the amount of insoluble protein can be determined for 68 flours per day. This represents a substantial improvement over the 14 samples/day (2 controls plus 12 unknowns) currently possible with SE-HPLC. Recent studies suggest that use of a Phenomenex BIO-SEP 4000 column and a flow rate of 2mL/min can reduce the run-time from 80 min to 20 min per sample equating to 48 samples/day (Larroque and Bekes, 2000), however, the cost of SE-HPLC is still prohibitive.

6.4 CONCLUSION

Market demand for wheat with different dough qualities requires that wheat breeders select appropriate lines in early generations of cultivar development. To do this it is imperative that there are rapid small-scale screening tests available. The Turbidity assay clearly has considerable potential as an early generation, quality prediction test in wheat breeding programs which, when compared to traditional rheological tests, allows for a large number of samples with limited seed supplies to be screened.

CHAPTER 7

PREDICTING PROTEIN QUALITY OF WILD WHEAT RELATIVES USING SE-HPLC AND THE TURBIDITY ASSAY

7.1 INTRODUCTION

Modern hexaploid bread wheat (*Triticum aestivum*) is composed of three genomes, A, B and D, derived from the hybridisation of three different but evolutionary related grass ancestors (Simmonds, 1989). While bread wheat has a relatively narrow genetic base, the wild and primitive wheat relatives contain a high degree of genetic diversity and are considered to be a useful source of germplasm for increasing genetic variability and the improvement of cultivated wheat (Sears, 1981). In addition to desirable disease and agronomic traits, wheat progenitors are highly polymorphic for gluten proteins and are likely to carry novel glutenin alleles that would exert positive effects on the bread making quality of bread wheat (Pena *et al.*, 1996).

The MWD of protein has a major influence on dough rheology. Possible ways to increase the MWD of protein in wheat cultivars would include the selection of glutenins alleles known to behave as chain extenders such as *Glu-D1d* (5+10), with increased levels of expression such as *Glu-B1al* (7+8*) and/or to increase the number of glutenin subunits encoded. For the latter approach, *Triticum turgidum* ssp. *dicoccoides* (2n = 4x = 28; AABB) would be useful because unlike hexaploid varieties, some *T. dicoccoides* accessions express both *Glu-A1* x- and y-type subunits (Levy *et al.*, 1985; Nevo and Payne, 1987; Levy and Feldman 1988; Ciaffi *et al.*, 1992 and 1993; Lafiandra *et al.*, 1993b). This study investigated the genetic variation in HMW-GS composition and protein quality attributes present in 335 accessions of *T. dicoccoides*. This was done to identify new *Glu-1* alleles that have the potential to improve the dough quality of Australian germplasm.

The acquisition of protein characteristics that make wheat suitable for bread production appears to have coincided with the introgression of the D-genome (Simmonds, 1989). The diploid donor of the D genome to bread wheat and other hexaploid subspecies (*spelta*, *vavilovii*, *macha*, *compactum* and *sphaerococcum*) is generally accepted to be *T. tauschii*. Two subspecies of *T. tauschii* have been recognised, ssp. *eusquarrosa* and ssp. *strangulata*. Varietal classes in the former subspecies are var. *typica*, *meyeri* and *anathera*, while var. *strangulata* is the only member of the latter subspecies and considered the most likely D-genome donor to hexaploid wheat (Lagudah and Halloran 1988). The D-genome of *T. tauschii* has greater variation in HMW-GS composition than the D-genome of bread wheat (Lagudah and Halloran, 1988; Ciaffi *et al.,* 1992; Williams *et al.,* 1993). Since HMW-GS variants controlled by the *Glu-D1* locus in bread wheat are strongly associated with gluten strength and bread making quality, the same can be expected to occur for HMW-GS variants of wheat lines carrying a 1DL chromosome of *T. tauschii* (Pena *et al.,* 1996).

To determine the protein quality attributes associated with the D-genome progenitor, 66 accessions of *T. tauschii* were analysed. Further, the production of primary synthetic hexaploids by colchicine treatment of seedlings derived from crosses between *T. turgidum* var. *durum* (AABB) and *T. tauschii* (DD) has also proven to be an excellent mechanism for investigating the genetic variability in the latter diploid species (Mujeeb-Kazi *et al.*, 1996; Villareal *et al.*, 1996; Pena *et al.*, 1996). This germplasm would also enable a direct breeding methodology to be employed for introgressing desirable alleles that are present in *T. tauschii* accessions. Accordingly, 148 synthetic hexaploids were analysed, not to determine the genetic variation, but to identify accessions with favourable protein quality attributes. Of the 148 accessions, 61 were primary synthetic hexaploids, while the remaining 87 were derived bread wheat lines which contain at least one synthetic hexaploid in their pedigree (derived synthetic hexaploids).

7.2 *Materials and Methods*

The genetic material (*T. dicoccoides*, *T. tauschii* and primary synthetic hexaploids and their derivatives) analysed in this chapter is described in more detail in Chapter 3. The methods used to conduct this work include SDS-PAGE (uniform and gradient) and SE-HPLC to characterise glutenin subunit banding patterns and determine %UPP, respectively (Chapter 3). Development of the Turbidity assay, as described in Chapter 6, enabled its use here to predict the protein quality (%IP) of *T. dicoccoides* accessions.

7.3 *Results and Discussion*

7.3.1 *Genetic variability and protein quality screening of the A- and B-genomes*

7.3.1.1 *Triticum dicocccoides*

The genetic variability observed in the *T. dicoccoides* accessions was extensive with 63 HMW-GS patterns identified (Table 7-1) and reported as GluA1.B1a through to GluA1.B1bk. As seven of these HMW-GS patterns appeared to be mixtures (Glu-A1.B1o, Glu-A1.B1u, Glu-A1.B1ar, Glu-A1.B1as, Glu-A1.B1at, Glu-A1.B1ax and Glu-A1.B1bk) on the basis of the number of subunits, there was a total of 56 different HMW-GS patterns. The most common HMW-GS patterns present were GluA1.B1b and Glu-A1.B1i and these were observed in 21% and 12.6% of the *T. dicoccoides* population, respectively. Of the other 54 HMW-GS patterns, 18 were present at frequencies of between 1% and 10%, while the remaining 36 patterns occurred in < 1% of the population (Table 7-1).

Numerous crosses were made between *T. dicoccoides* accessions containing different HMW-GSs which resulted in ≈50 F_1 derived lines, but the subsequent germination protocol was unsuccessful. Hence preventing the preferred method of assigning HMW-GS pairs to either the A- or B-genomes based on inheritance studies from being applied.

Consequently, identification of x- and y-type subunits from the A- and B-genomes was based on the contrasting SDS-PAGE mobilities of HMW-GSs in *T. dicoccoides* reported by Lafiandra and co-workers in 1993 which was essentially 1Ax > 1Bx >1By > 1Ay (Chapter 2, pg 21). Bread wheats were used as internal reference standards for SDS-PAGE analysis. Individual subunits observed here have been numbered in a fashion analogous to that of Payne *et al.* (1987) for *T. aestivum* and new allelic designations with a superscript ^{d} were tentatively assigned (Table 7-1 and Appendix V).

In total there were 49 HMW-GSs including 15 *Glu-A1* x-type and 8 *Glu-A1* y-type subunits, while at the *Glu-B1* locus 15 x-type and 11 y-type subunits were identified. In addition to the null allele (*Glu-A1c*^{d}) which was present in 5 of the 56 HMW-GS patterns, the HMW-GSs encoded by the *Glu-A1* locus in *T. dicoccoides* combined to produce a further 22 different alleles making the total number of *Glu-A1* alleles indentified here 23. Of the 22 alleles that encoded a protein product, 12 comprised only a single x-type subunit while the remaining 10 alleles expressed both 1Ax and 1Ay subunits. The majority of HMW-GS patterns (62.5%) contained alleles which encoded a single x-type subunit, whereas the null allele and alleles encoding both 1Ax and 1Ay subunits accounted for 8.9% and 28.6%, respectively. At the *Glu-B1* locus 32 different alleles were identified, these alleles usually consisted of both x- and y- type subunits but there were also alleles which encoded either no subunits (*Glu-B1t*^{d}), a single x-type subunit (*Glu-B1c*^{d}, *Glu-B1d*^{d}, *Glu-B1r*^{d}, *Glu-B1x*^{d}, *Glu-B1ab*^{d}, *Glu-B1ac*^{d}) or a single y-type subunit (*Glu-B1k*^{d}).

Possible associations with the HMW-GS patterns and protein quality attributes were initially investigated using the Turbidity assay (%IP). Results of the turbidimetric screening revealed that a large variation in %IP was present in the *T. dicoccoides* accessions, ranging from 27.7% to 64.9% (data not shown). ANOVA on this data was

HMW-GS Patterns					*Glu-A1*[d]		*Glu-B1*[d]	
Pattern	Standard [a]	Frequency	%IP [b]	lsd [c]	Subunits	Allele	Subunits	Allele
GluA1.B1a	AUS 21322	0.027	55.4		12x	*a*	29x+34y	*a*
GluA1.B1b	AUS 21318	0.210	53.4		12x+42y	*b*	26x+34y	*b*
GluA1.B1c	AUS 21312	0.045	51.1	*	null	*c*	26x+34y	*b*
GluA1.B1d	AUS 21288	0.027	43.3	***	null	*c*	25x	*c*
GluA1.B1e	AUS 21311	0.006	44.6	***	11x+42y	*d*	20x	*d*
GluA1.B1f	AUS 21306	0.006	50.5	*	11x+42y	*d*	26x+37y	*e*
GluA1.B1g	AUS 21317	0.006	47.4	**	12x+45y	*e*	26x+34y	*b*
GluA1.B1h	AUS 21420	0.006	52.7		12x+42y	*b*	26x+33y	*f*
GluA1.B1i	AUS 21426	0.126	53.2		11x+42y	*d*	26x+34y	*b*
GluA1.B1j	AUS 21432	0.030	53.9		6x+48y	*f*	29x+39y	*g*
GluA1.B1k	AUS 21433	0.003	45.1	***	6x+48y	*f*	23x+40y	*h*
GluA1.B1l	AUS 21436	0.012	52.4		12x+42y	*b*	24x+41y	*i*
GluA1.B1m	AUS 21447	0.012	51.6	*	8x	*g*	29x+39y	*g*
GluA1.B1n	AUS 21443	0.012	52.0	*	15x+44y	*h*	21x+36y	*j*
GluA1.B1o	-	-	-		-	-	-	-
GluA1.B1p	AUS 21453	0.054	60.8		5x	*i*	24x+41y	*i*
GluA1.B1q	AUS 21463	0.012	39.7	***	14x	*j*	35y	*k*
GluA1.B1r	AUS 21478	0.018	53.9		12x+42y	*b*	30x+35y	*l*
GluA1.B1s	AUS 21479	0.009	53.2		13x	*k*	24x+41y	*i*
GluA1.B1t	AUS 21418	0.003	50.4	*	14x	*j*	30x+35y	*l*
GluA1.B1u	-	-	-		-	-	-	-
GluA1.B1v	AUS 21484	0.006	50.4	*	14x	*j*	24x+41y	*i*
GluA1.B1w	AUS 21497	0.015	47.3	**	5x	*i*	24x+34y	*m*
GluA1.B1x	AUS 21501	0.009	48.1	**	6x	*l*	28x+33y	*n*
GluA1.B1y	AUS 21504	0.018	51.7	*	null	*c*	26x+33y	*o*
GluA1.B1z	AUS 21515	0.003	49.1	**	7x	*m*	20x+37y	*p*
GluA1.B1aa	AUS 21516	0.003	50.5	*	5x	*i*	20x+37y	*p*
GluA1.B1ab	AUS 22282	0.006	43.4	***	8x	*g*	24x+34y	*m*
GluA1.B1ac	AUS 22281	0.003	43.0	***	7x	*m*	15x+36y	*q*
GluA1.B1ad	AUS 23140	0.018	51.8	*	5x	*i*	29x+34y	*a*
GluA1.B1ae	AUS 22283	0.003	46.2	***	3x	*n*	15x+36y	*q*
GluA1.B1af	AUS 21519	0.012	49.5	**	1x	*o*	24x+34y	*m*
GluA1.B1ag	AUS 23142	0.003	52.8		null	*c*	20x+37y	*p*
GluA1.B1ah	AUS 23145	0.003	48.4	**	1x	*o*	29x	*r*
GluA1.B1ai	AUS 23149	0.015	51.9	*	14x	*j*	21x+39y	*s*
GluA1.B1aj	AUS 23153	0.006	29.4	***	7x	*m*	null	*t*
GluA1.B1ak	AUS 23144	0.021	47.0	***	7x	*m*	25x	*c*
GluA1.B1al	AUS 27008	0.003	39.5	***	14x	*j*	20x	*d*
GluA1.B1am	AUS 27013	0.003	47.6	**	10x	*p*	18x+33y	*u*
GluA1.B1an	AUS 22285	0.006	45.9	***	3x	*n*	24x+34y	*m*
GluA1.B1ao	AUS 21502	0.006	49.0	**	6x	*l*	27x+32y	*v*
GluA1.B1ap	AUS 27017	0.003	33.8	***	7x+49y	*q*	17x+31y	*w*
GluA1.B1aq	AUS 21517	0.003	48.4	**	7x	*m*	24x+41y	*i*
GluA1.B1ar	-	-	-		-	-	-	-
GluA1.B1as	-	-	-		-	-	-	-
GluA1.B1at	-	-	-		-	-	-	-
GluA1.B1au	AUS 26988	0.009	46.1	***	7x	*m*	24x	*x*
GluA1.B1av	AUS 23148	0.012	50.3	*	5x	*i*	21x+35y	*y*
GluA1.B1aw	AUS 23147	0.003	55.8		5x	*i*	24x	*x*
GluA1.B1ax	-	-	-		-	-	-	-
GluA1.B1ay	AUS 23143	0.003	57.1		7x	*m*	29x+34y	*a*
GluA1.B1az	AUS 23140	0.015	47.8	**	7x	*m*	24x+34y	*m*
GluA1.B1ba	AUS 14897	0.003	52.5	*	4x+43y	*r*	19x+31y	*z*
GluA1.B1bb	AUS 17639	0.003	27.7	***	4x+49y	*s*	16x+31y	*aa*
GluA1.B1bc	AUS 23150	0.003	36.2	***	4x+46y	*t*	19x+31y	*z*
GluA1.B1bd	AUS 15820	0.003	37.7	***	8x+47y	*u*	27x	*ab*
GluA1.B1be	AUS 17967	0.003	31.7	***	9x	*v*	22x	*ac*
GluA1.B1bf	AUS 27025	0.003	49.6	**	2x	*w*	21x+36y	*j*
GluA1.B1bg	AUS 27022	0.009	43.5	***	null	*c*	24x+34y	*m*
GluA1.B1bh	AUS 27028	0.003	45.0	***	8x	*g*	21x+34y	*ad*
GluA1.B1bi	AUS 27029	0.003	43.7	***	7x	*m*	20x+33y	*ae*
GluA1.B1bj	AUS 23160	0.003	44.9	***	5x	*i*	21x+33y	*af*
GluA1.B1bk	-	-	-		-	-	-	-

[a] Accession used as a standard for a particular allele
[b] ANOVA predicted means for %IP
[c] * = p≤0.001, ** = p≤0.01, ***p≤0.05

Table 7-1 %IP and HMW-GSs associated with GluA1.B1 patterns in *T. dicoccoides* and their provisional allelic designations. Lsd levels for mean %IP are from comparisons with GluA1.B1p which had the highest mean %IP.

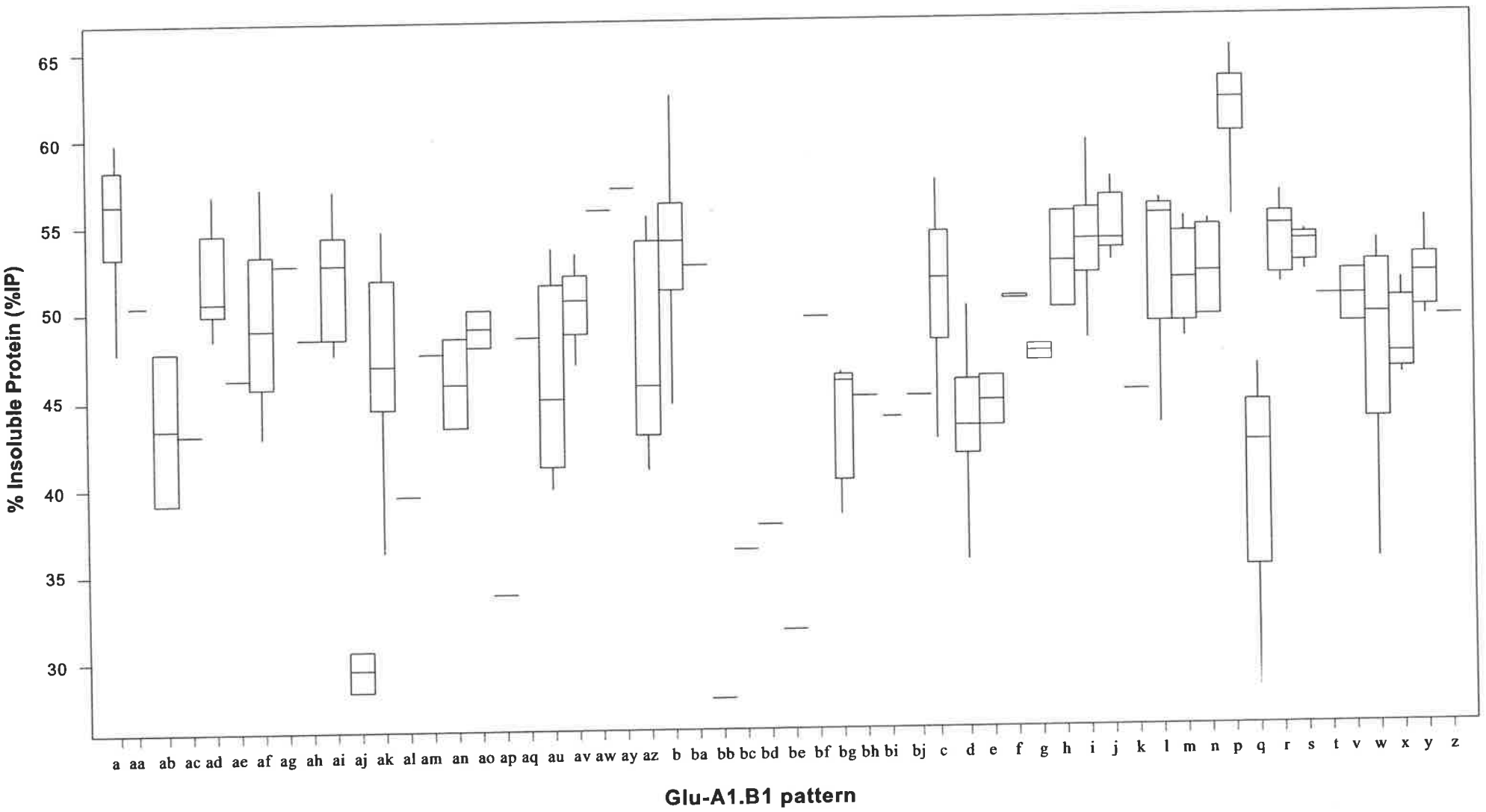


Figure 7-1 Boxplot of the HMW-GS patterns in *T. dicoccoides*. The box spans the interquartile range of mean %IP's so that the middle 50% of the data lie within the box where a line indicates the median, the bars beyond the ends of the box extend as far as the minimum and maximum values.

conducted to obtain %IP means for each of the 56 different GluA1.GluB1 patterns. Least significant differences compared to GluA1.B1p, which had the highest mean % IP, from the ANOVA are reported (Table 7-1) while the median, range and 50% interquartile of GluA1.B1 patterns are presented diagrammatically in Figure 7-1.

Results regarding the relationship between HMW-GS patterns and %IP were not as expected since it was assumed that accessions which expressed both 1Ax and 1Ay subunits, rather than a single 1Ax subunit, would have a higher %IP. However, the accession with the highest mean %IP possessed the HMW-GS pattern GluA1.B1p which is associated with only an x-type HMW-GS encoded at *Glu-A1*. In addition, the whole interquartile for %IP was higher for GluA1.GluB1p than for any other HMW-GS pattern. From this data it is difficult to pinpoint whether the *Glu-A1* or *Glu-B1* allele is responsible for the favourable protein characteristics. This is further complicated by the fact that LMW-GS composition was not investigated. However, it appears that *Glu-B1i*^{d} (24x + 41y) is most likely responsible. Of the four other patterns that contain *Glu-B1i*^{d}, two (GluA1.B1l and GluA1.B1s) did not have a mean %IP significantly different from GluA1.B1p. Whereas, of the six patterns which possess the *Glu-A1i*^{d} allele only one, GluA1.B1aw, did not have a mean %IP significantly less than observed for GluA1.B1p, and this pattern has the 1Bx24 subunit, the same 1Bx subunit identified in GluA1.B1p. If the high proportion of insoluble protein is conveyed by the *Glu-B1i*^{d} allele then the %IP intrinsically found in *T. dicoccoides* lines that possess the GluA1.B1p HMW-GS pattern could be increased by substituting *Glu-A1i*^{d} with an allele that encodes both x- and y-type HMW-GSs (eg. *Glu-A1b*^{d}).

It was not surprising that both the HMW-GS patterns that possessed only one subunit (GluA1.GluB1d and GluA1.B1aj) had significantly ($P \leq 0.001$) lower %IP than compared to GluA1.B1p. Other GluA1.B1 patterns that had a significantly lower ($P \leq 0.001$) %IP values

include 55% of the patterns associated with two HMW-GSs (6 out of 11), 28% of patterns with three HMW-GSs (8 out of 29) and 29% of HMW-GS patterns with four subunits (4 out of 14). Interestingly, of the four GluA1.B1 patterns associated with four HMW-GSs and low %IP (GluA1.B1k, GluA1.B1ap, GluA1.B1bb and GluA1.B1bc) two contained the 1Ax4 ^{d} subunit, two contained 1Ay49 ^{d} and two contained the 1By31 ^{d} subunit. Even though no glutenin alleles were common to all four of these HMW-GS patterns *T. dicoccoides* alleles associated with the subunits 1Ax4 ^{d}, 1Ay49 ^{d} and 1By31 ^{d} should be avoided. These results also indicate that not all *T. dicoccoides Glu-A1* alleles that encode both x- and y-type HMW-GS subunits would positively contribute to dough strength.

To confirm the findings of the Turbidity assay, SE-HPLC was conducted on a subset of 90 accessions that represented the range of %IP present in the *T. dicoccoides* population. The subset included those with a %IP in the top 20% (67 accessions) and 23 other accessions ranging to the one with the lowest %IP. As shown in Figure 7-2, the linear regression between %IP and %UPP was highly significant and positively correlated ($r = 0.95$, $P \leq 0.001$). It also confirmed the high %IP observed for GluA1.B1p which was present in 14 out of the top 20 accessions for %UPP, including the first 11 (Table 7-2). Clearly, this is

Rank	AUS#	%UPP	%IP	HMW-GS pattern	Rank	AUS#	%UPP	%IP	HMW-GS pattern
1	21457	69.4	64.8	Glu-A1.B1p	11	21469	65.4	60.5	Glu-A1.B1p
2	21461	68.5	63.4	Glu-A1.B1p	12	21416	65.2	61.1	Glu-A1.B1b
3	21455	68.4	64.4	Glu-A1.B1p	13	21333	65.1	60.4	Glu-A1.B1b
4	21462	68.0	64.0	Glu-A1.B1p	14	21326	64.8	62.3	Glu-A1.B1b
5	21464	67.2	61.9	Glu-A1.B1p	15	21465	63.7	59.8	Glu-A1.B1p
6	21474	67.0	62.7	Glu-A1.B1p	16	21324	63.6	58.9	Glu-A1.B1b
7	21460	66.5	61.7	Glu-A1.B1p	17	21268	63.5	59.8	Glu-A1.B1a
8	21454	66.5	63.1	Glu-A1.B1p	18	21257	63.5	59.5	Glu-A1.B1i
9	21456	66.3	62.7	Glu-A1.B1p	19	21467	63.5	60.4	Glu-A1.B1p
10	21458	66.0	62.4	Glu-A1.B1p	20	21334	63.1	59.9	Glu-A1.B1b

Table 7-2 %UPP (SE-HPLC), %IP (Turbidity asay), and HMW-GS protein banding patterns (SDS-PAGE) of 20 *T. dicoccoides* accessions with the highest %UPP.

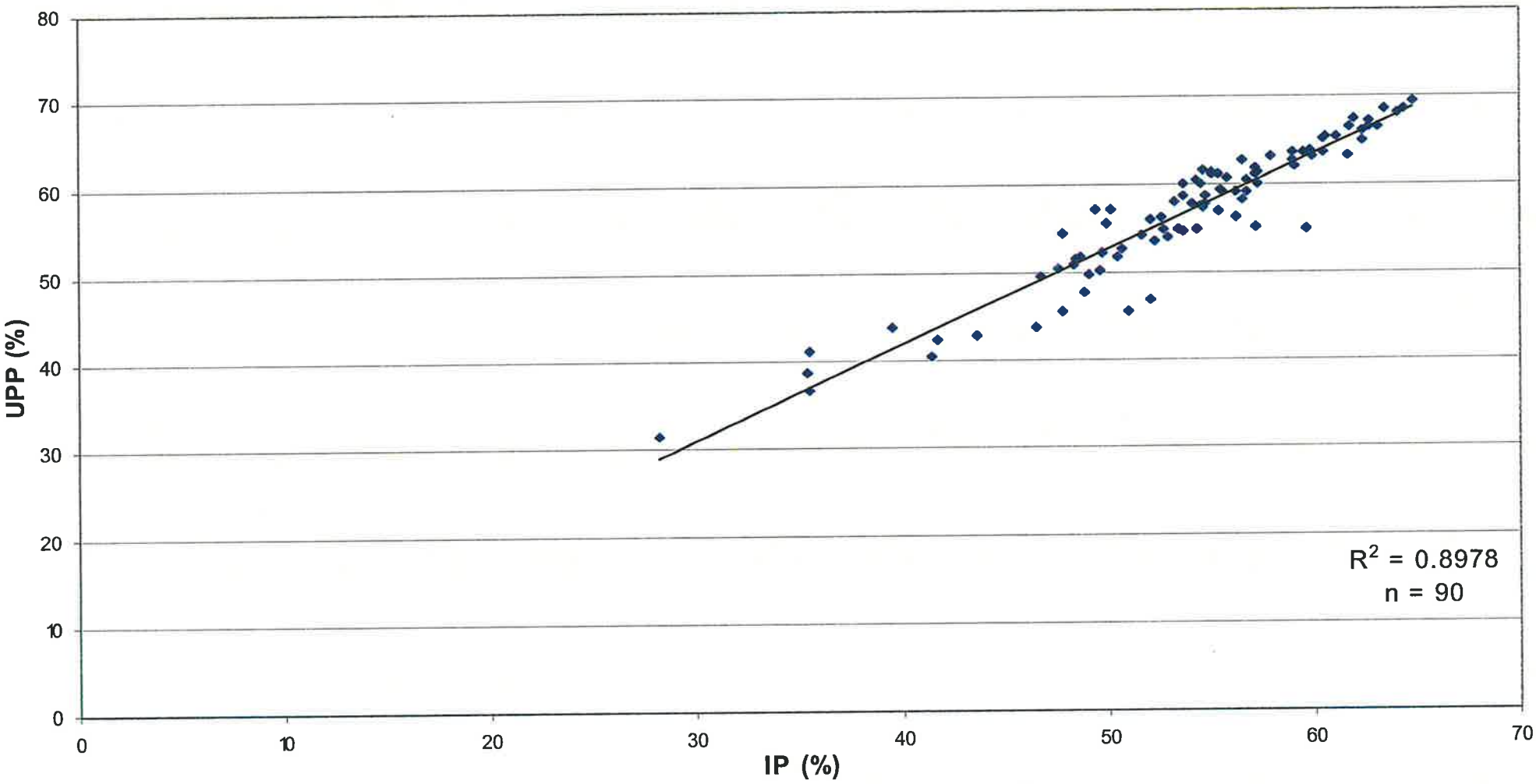


Figure 7-2 Linear regression between %IP and %UPP of 90 selected *T. dicoccoides* accessions; correlation coefficient r = 0.95, P ≤ 0.001.

sufficient evidence to warrant further investigation aimed at elucidating the effect of the *Glu-A1i* ^{d} and *Glu-B1i* ^{d} alleles, either individually or combined, on dough rheological parameters and their potential to improve bread making quality.

7.3.2 Protein quality screening of the D-genome progenitor

7.3.2.1 Triticum tauschii

%UPP values obtained for the 66 *T. tauschii* accessions was variable and ranged from 25.4% to 68.0% (Table 7-3). ANOVA of this data was used to compare the mean %UPP for the *T. tauschii* subspecies, ssp. *eusquarossa* var. *typica* (44 accessions) and *meyeri* (5 accessions) and ssp. *strangulata* var. *strangulata* (17 accessions). No relationship was shown between %UPP and *T. tauschii* varietal classes (Figure 7-3).

Where quantity of seed sample permitted, protein of 12 of the *T. tauschii* accessions with the highest %UPP was extracted and analysed using SDS Gradient PAGE (Figure 7-4A and B) HMW-GSs (*Glu-D1* ^{t}), the same subunit pair was present in accessions AUS 24163

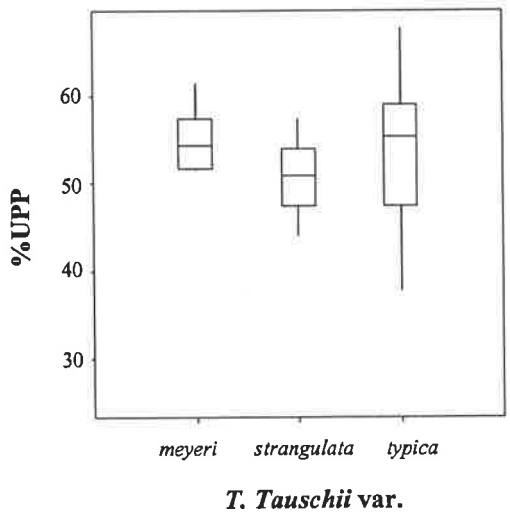


Figure 7-3 Boxplot of %UPP statistics for *T. tauschii* accessions. The box spans the interquartile range of mean %IP's so that the middle 50% of the data lie within the box where a line indicates the median, the bars beyond the ends of the box extend as far as the minimum and maximum observed values.

#	Accession	Varietal class	Origin	%UPP	#	Accession	Varietal class	Origin	%UPP
1	AUS 24164	*typica*	Iran	67.66	34	AUS 24150	*meyeri*	Iran	54.24
2	AUS 24155	*typica*	Iran	66.19	35	AUS 24186	*typica*	Iran	53.61
3	AUS 24163	*typica*	Iran	64.10	36	AUS 24187	*typica*	Iran	53.38
4	AUS 24167	*typica*	Iran	63.09	37	AUS 24144	*strangulata*	Iran	52.77
5	AUS 24165	*typica*	Iran	62.72	38	AUS 24133	*strangulata*	Iran	52.75
6	AUS 24195	*typica*	Iran	62.39	39	AUS 24126	*strangulata*	Iran	52.58
7	AUS 24200	*meyeri*	Iran	61.35	40	AUS 24201	*typica*	Iran	52.20
8	AUS 24171	*typica*	Iran	61.10	41	AUS 24158	*meyeri*	Iran	51.78
9	AUS 24197	*typica*	Iran	60.51	42	AUS 24159	*meyeri*	Iran	51.52
10	AUS 24122	*typica*	Iran	59.93	43	AUS 24128	*strangulata*	Iran	51.09
11	AUS 24154	*typica*	Iran	59.78	44	AUS 24142	*strangulata*	Iran	50.56
12	AUS 24169	*typica*	Iran	59.30	45	AUS 24130	*strangulata*	Afghanistan	50.07
13	AUS 24170	*typica*	Iran	58.61	46	AUS 24140	*strangulata*	Iran	49.99
14	AUS 24135	*typica*	Iran	58.60	47	AUS 24157	*typica*	Iran	49.73
15	AUS 24172	*typica*	Iran	58.42	48	AUS 24129	*strangulata*	Iran	49.00
16	AUS 24194	*typica*	Iran	58.30	49	AUS 24153	*typica*	Iran	48.67
17	AUS 24131	*strangulata*	Iran	57.28	50	AUS 24162	*typica*	Iran	48.61
18	AUS 24174	*typica*	Iran	57.24	51	AUS 24132	*strangulata*	Iran	47.53
19	AUS 24196	*typica*	Iran	57.06	52	AUS 24147	*strangulata*	Iran	47.34
20	AUS 24202	*meyeri*	Iran	56.12	53	AUS 24148	*typica*	Iran	46.22
21	AUS 24193	*typica*	Iran	55.88	54	AUS 24166	*typica*	Iran	46.12
22	AUS 24160	*typica*	Iran	55.80	55	AUS 24134	*typica*	Iran	45.68
23	AUS 24146	*strangulata*	Iran	55.72	56	AUS 24152	*typica*	Iran	45.50
24	AUS 24192	*typica*	Iran	55.66	57	AUS 24199	*typica*	Iran	44.98
25	AUS 24124	*typica*	Iran	55.40	58	AUS 24145	*strangulata*	Iran	44.66
26	AUS 24190	*typica*	Iran	55.40	59	AUS 24156	*typica*	Iran	44.43
27	AUS 24188	*typica*	Iran	55.39	60	AUS 24141	*strangulata*	Iran	43.99
28	AUS 24125	*typica*	Iran	55.20	61	AUS 24161	*typica*	Iran	42.41
29	AUS 24143	*strangulata*	Iran	55.16	62	AUS 24198	*typica*	Iran	41.81
30	AUS 24139	*strangulata*	Iran	55.02	63	AUS 24173	*typica*	Iran	39.63
31	AUS 24168	*typica*	Iran	54.90	64	AUS 24138	*typica*	Iran	37.66
32	AUS 24189	*typica*	Iran	54.88	65	AUS 24137	*typica*	Iran	27.63
33	AUS 24191	*typica*	Iran	54.84	66	AUS 24127	*strangulata*	Iran	25.42

Table 7-3 Listing of 66 *T. tauschii* accessions, their geographical origin, varietal class and SE-HPLC %UPP results.

(Figure 7-4B lanes 5 & 6), AUS 24165 (lanes 9 & 10) and AUS 24122 (lanes 19 & 20) while a second HMW- GS allele that was present in more than one accession was in AUS 24169 (lanes 23 & 24), AUS 24170 (not shown) and AUS 24135 (lanes 25 & 26). Similarly, two LMW-GS alleles (*Glu-D3* ^{t}) were present in more than one accession, one in the accessions AUS 24164 (lanes 1 & 2), AUS 24163 (lanes 5 & 6) and AUS 24195 (lanes 11 & 12) while the other allele was present in AUS 24197 (lanes 21 & 22) and AUS 24122 (lanes 23 & 24).

The presence of the *Glu-D3* ^{t} alleles in more than one accession is supported by the similar gliadin banding patterns since there is a close genetic linkage between *Gli-D1* and *Glu-D3* alleles. This can be seen in Figure 7-4 (A), for example lanes 1, 2, 5, 6, 11 and 12 possess gliadin subunit bands with similar electrophoretic mobilities, this is also true for lanes 21, 22, 23, and 24. The presence of these subunits in more than one accession with high %UPP suggests that the alleles which encode them may convey desirable protein quality attributes.

From comparisons between the HMW-GS banding patterns for the *T. tauschii* accessions and the standard bread wheat varieties Jabiru and Wilgoyne (Figure 7-4B, lanes 15 and 16, respectively) it was evident that the 1Dx5 ^{t} subunit did not feature in the lines with the highest %UPP. This is not particularly surprising since 1Dx5 ^{t} does not contain the extra cysteine residue that is present in the 1Dx5 subunit of bread wheat (Mackie *et al.*, 1996a) and highlights the need to exercise caution when crossing wild wheat species and bread wheat cultivars to avoid selection of the subunits 1Dx5 ^{t} + 1Dy10 ^{t} from *T. tauschii* instead of the *Glu-D1d* bread wheat allele.

It appears that some *T. tauschii* accessions with high %UPP and intense staining of their HMW-GS, namely AUS 24155 and AUS 24167 (Figure 7-5, lanes 3 & 4 and lanes 7 & 8,

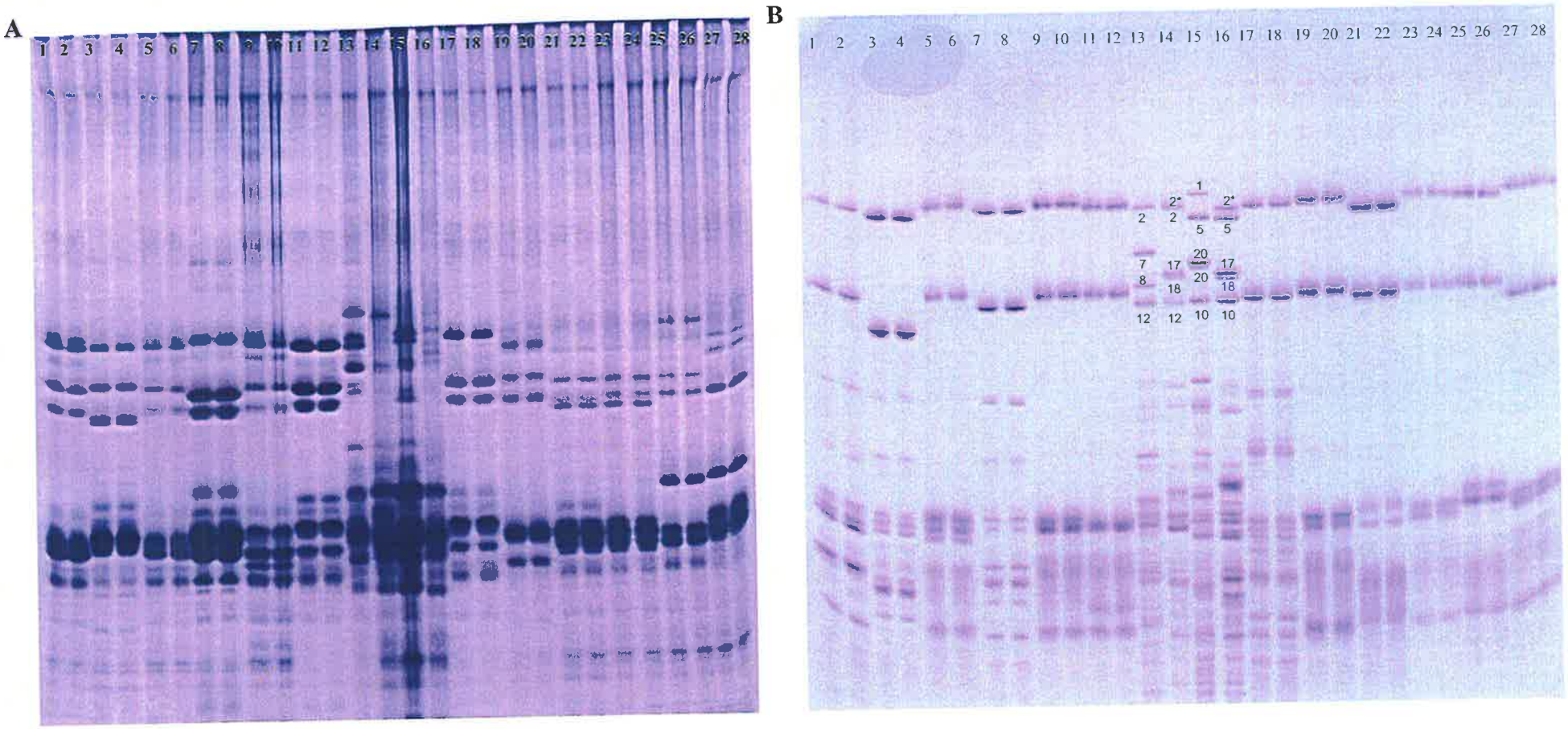


Figure 7-4 SDS Gradient PAGE (8-12%) of *T. tauschii* accession with high %UPP. **A** Gliadins. **B** Glutenins. Lane 1-2, AUS 24164; Lanes 3-4, AUS 24155; Lanes 5-6, AUS 24163; Lanes 7-8, AUS 24167; Lanes 9-10, AUS 24165; Lanes 11-12, AUS 24195; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-18, AUS 24200; Lanes 19-20, AUS 24171; Lanes 21-22, AUS 24197; Lanes 23-24, AUS 24122; Lanes 25-26, AUS 24154; Lanes 27-28, AUS 24169; Lane 27-28, AUS 24170; Lanes 27-28, AUS 24135; Lanes 27-28, AUS 24172.

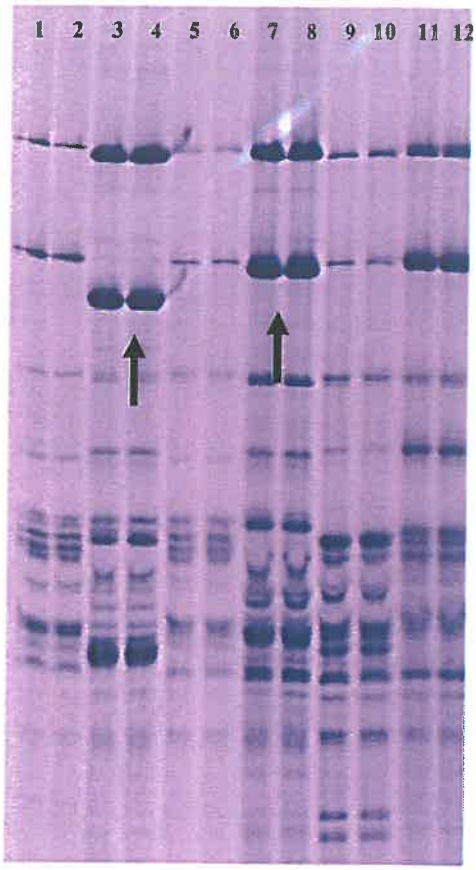


Figure 7-5 SDS Gradient PAGE (8-12%) of glutenins from *T. tauschii* accession with high %UPP. Lane 1-2, AUS 24164; Lanes 3-4, AUS 24155; Lanes 5-6, AUS 24163; Lanes 7-8, AUS 24167; Lanes 9-10, AUS 24165; and Lanes 11-12, AUS 24195. Arrows indicate possible increased levels of HMW-GS expression.

respectively), may be over-expressing *Glu-D1* ^{t} encoded subunits since the staining intensity of LMW-GS bands in Figure 7-5 does not indicate that any samples were over-loaded. However, even though Coomassie Blue staining procedures are common place, drawbacks, primarily related to inconsistencies in destaining, do exist. More sensitive staining procedures are available and these may have provided more information (Marchylo, pers. comm.). Notwithstanding, if these accessions do possess increased levels of HMW-GS expression, they could have a large impact on future cultivar development in Australian wheat breeding programs, especially if the effect of *T. tauschii* over-expression is similar to that conveyed by the over-expression of the 1Bx7 subunit (*Glu-B1al*, refer Chapter 5) in hexaploid wheat.

7.3.2.2 Synthetic Hexaploids

SE-HPLC analysis showed that the %UPP in derived synthetic hexaploid lines ranged from 37.1% to 68.0% and from 33.2% to 61.5% for the primary synthetic hexaploids (data not shown). Figure 7-6 and Figure 7-7 show the gliadin (A) and glutenin (B) subunit banding patterns on SDS-PAGE associated with the derived and primary synthetic hexaploid lines with the highest %UPP, respectively. SDS-PAGE enabled comparisons of HMW-GS electrophoretic mobilities between accessions so that commonly expressed HMW-GSs among the selected lines could be identified, as indicated in Table 7-4 by Roman numerals. Four out of the five derived synthetic hexaploids (AUS 27767, AUS 27737, AUS 27736 and AUS 27733) with the highest %UPP had similar *Glu-A1* (I) and *Glu-D1* (i) HMW-GSs (Table 7-4). It is possible that the *Glu-A1* (I) subunit is actually 1Ax2* based on the electrophoretic mobility of the 1Ax subunit in these lines (Figure 7-6, lanes 1, 2, 5, 6, 7, 8, 9 and 10) and Gabo (Figure 7-6, lane 14). This suggests that the *Glu-D1* (i) allele is most likely responsible for the high %UPP observed in these lines. The *Glu-D1* (i) allele is derived from a *T. tauschii* accession since it was also present in two of the selected primary synthetic lines, AUS 26808 and AUS 26811 (Figure 7-7 Lanes 5, 6, 19 and 20). If the *Glu-A1*x subunit is a 2*, the 1Dx subunit associated with *Glu-D1* (i) can be described as having an electrophoretic mobility between 1Dx2 and 1Dx5 of bread wheat and a 1Dy subunit slightly less mobile than the 1Dy10 subunit of bread wheat.

The remaining derived synthetic hexaploid (AUS 27724) of the five accessions with the highest %UPP was found to possess the *Glu-B1al* allele and its over-expression of 1Bx7 was confirmed using RP-HPLC as described in Chapter 3. The *Glu-B1al* allele present in AUS 27724 was donated by the Mexican bread wheat cultivar, Flycatcher (Chapter 5). Based on SDS Gradient PAGE analysis, the *Glu-B1* subunits in the other selected lines are

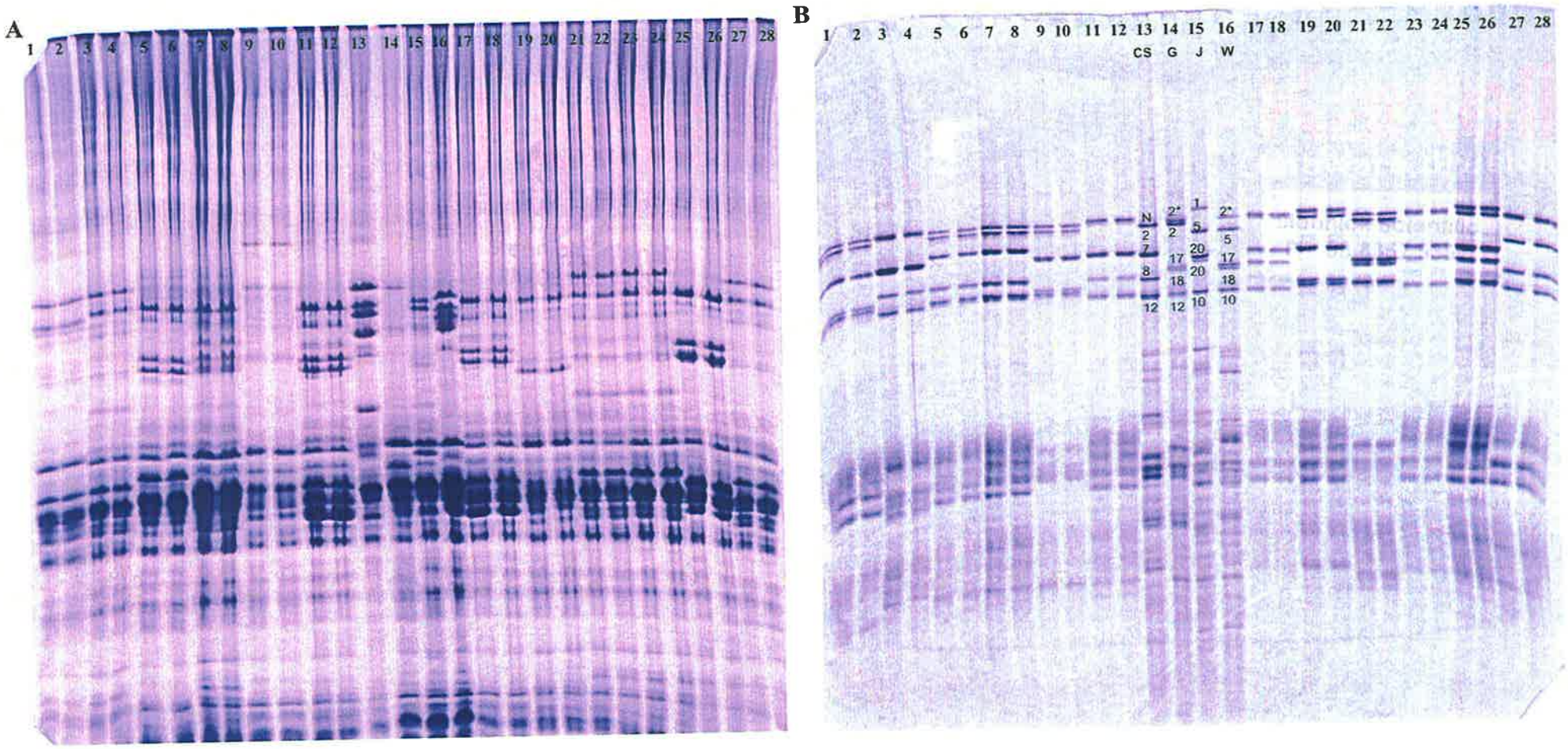


Figure 7-6 SDS Gradient PAGE (8-12%) of derived synthetic hexaploid accessions with high %UPP. **A** Gliadins. **B** Glutenins. Lane 1-2, AUS 27767; Lanes 3-4, AUS 27724; Lanes 5-6, AUS 27737; Lanes 7-8, AUS 27736; Lanes 9-10, AUS 27733; Lanes 11-12, AUS 27768; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-18, 27762; Lanes 19-20, AUS 27730; Lanes 21-22, AUS 27738; Lanes 23-24, AUS 27769; Lanes 25-26, AUS 27743; Lanes 27-28, AUS 27765.

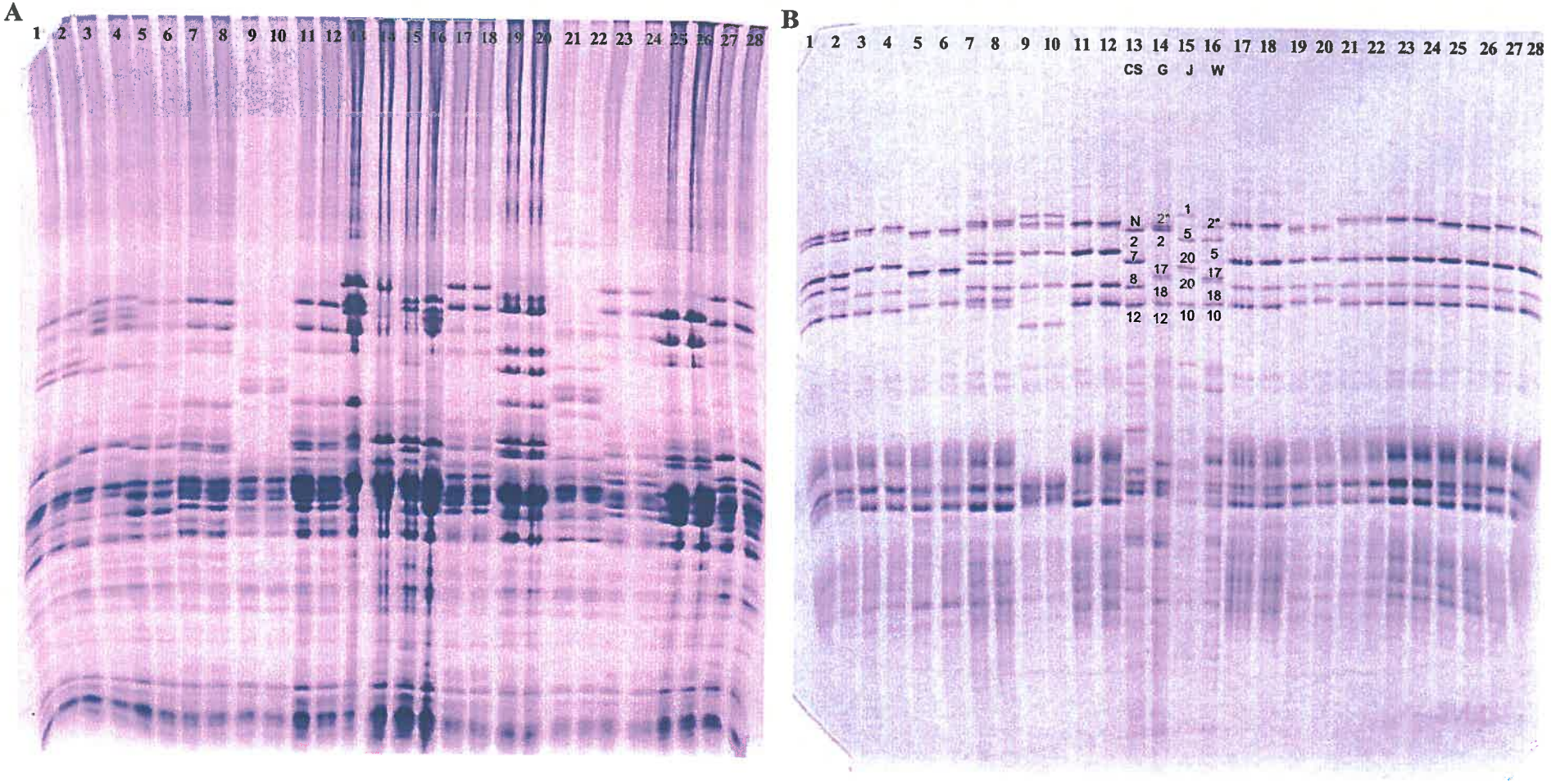


Figure 7-7 SDS Gradient PAGE (8-12%) of primary synthetic hexaploid accessions with high %UPP. **A** Gliadins. **B** Glutenins. Lane 1-2, AUS 26846; Lanes 3-4, AUS 26821; Lanes 5-6, AUS 26808; Lanes 7-8, AUS 26833; Lanes 9-10, AUS 26880; Lanes 11-12, AUS 26890; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-18, AUS 26881; Lanes 19-20, AUS 26811; Lanes 21-22, AUS 26852; Lanes 23-24, AUS 26876; Lanes 25-26, AUS 26809; Lanes 27-28, AUS 26822.

	Accession	%UPP	Pedigree	*Glu-A1*	*Glu-B1*	*Glu-D1*
Derived						
1	AUS 27767	68.0	BCN/4/68.111/RGB-U//WARD/3/AE.SQ(325)	I	7*+9	i
2	AUS 27724	66.8	CROC_1/AE.SQUARROSA(205)//2*FCT	II	7+8* ^{OE}	ii
3	AUS 27737	64.4	BCN/3/FGO/USA2111//AE.SQUARROSA(658)	I	6+8*	i
4	AUS 27736	64.2	BCN/3/68112/WARD//AE.SQUARROSA(369)	I	6+8*	i
5	AUS 27733	64.0	BCN*2//CROC_1//AE.SQUARROSA(886)	I	7*+9	i
6	AUS 27768	62.7	BCN//CETA/AE.SEARSII(34D)	II	7* +8?	ii
7	AUS 27762	62.2	BCN/6/68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQ(629)	II	13+16	ii
8	AUS 27730	62.1	BCN//CROC_1/AE.SQUARROSA(168)	III	7*+9	iii
9	AUS 27738	61.8	BCN//DOY1/AE.SQUARROSA(447)	I	17+18	i
10	AUS 27769	61.7	OPATA//SORA/AE.SQ(323)	II	13+16	ii
11	AUS 27743	61.3	ALTAR 84/AE.SQUARROSA(191)//OPATAT/6/CROC-1/AE.SQUARROSA(205)/5/....	III	13+16	iii
12	AUS 27765	61.3	OPATA//TK SN1081/AE.SQ(519)	II	6+8*	ii
Primary						
1	AUS 26846	61.5	CETA/AE.SQUARROSA(895)	2*	13+16	iv
2	AUS 26821	58.5	DVERD_2/AE.SQUARROSA(221)	N	7*+8	iv
3	AUS 26808	58.1	ALTAR_84/AE.SQUARROSA(193) – durum parent incorrect, most likely YAV	N	20+20	i
4	AUS 26833	57.4	CROC_1/AE.SQUARROSA(725)	---- segregating ----		
5	AUS 26880	57.4	DOY_1/AE.SQUARROSA(258)	1	6+8*	v
6	AUS 26890	57.4	YUK/AE.SQUARROSA(864)	N	6+8*	vi
7	AUS 26881	56.9	ALTAR_84/AE.SQUARROSA(502)	N	7*+8	vi
8	AUS 26811	56.8	ALTAR_84/AE.SQUARROSA(205)	N	7*+8	i
9	AUS 26852	55.8	SCA/AE.SQUARROSA(518)	N	7*+8	iv
10	AUS 26876	55.7	DVERD_2/AE.SQUARROSA(1027)	N	7*+8	iv
11	AUS 26809	55.6	ALTAR_84/AE.SQUARROSA(198)	N	7*+8	vi
12	AUS 26822	55.6	ALTAR_84/AE.SQUARROSA(221)	N	7*+8	iv

Table 7-4 Pedigrees and SDS-PAGE results for synthetic hexaploids (derived and primary) containing a high %UPP. Accessions followed by the same roman numeral possess similar glutenin subunits encoded at that locus. N denotes null; ^{OE} denotes over-expression (*Glu-B1al*).

also indicated in Table 7-4, however these do not always correspond with the HMW-GS alleles of the putative parents in the pedigree data obtained from the AWCC. This makes definitively indentifying HMW-GSs problematic since parental information is often used to facilitate the indentification of glutenin subunit composition. For example, the pedigree of AUS 27738 suggests that the *Glu-B1* subunits present should either be 7*+9 from Bacanora (bread wheat) or 6+8* from Decoy 1 (durum) but here the *Glu-B1* subunits were found to be the 17+18 subunit pair. Similarly, the primary synthetic AUS 26808 should possess the *Glu-B1* subunits 7*+8 based on its durum parent (Altar 84) but was found to have the 20+20 subunit pair instead. The most likely source of 20+20 (*Glu-B1e*) is Yavaros, another durum variety used extensively as a parent in the production of synthetic hexaploid lines at CIMMYT.

7.4 CONCLUSION

Clearly, undomesticated relatives of wheat are an important source for new glutenin genes that are not present in bread wheat. It is envisaged that *Glu-A1*, *Glu-B1* and *Glu-D1* alleles present in accessions identified in this study would, when introgressed into well adapted germplasm, increase the dough strength of Australian bread wheat cultivars. For *T. dicoccoides*, accessions that had the highest %UPP contained the HMW-GS pattern GluA1.B1p (*Glu-A1i* ^{d}, *Glu-B1i* ^{d}) followed by Glu-A1.B1b (*Glu-A1b* ^{d}, *Glu-B1b* ^{d}). Accordingly, crosses have been made using two *T. dicoccoides* accessions, AUS 21457 (GluA1.B1p), AUS 21416 (GluA1.B1b) and the Australian bread wheat cultivar, Aroona. Further backcrossing of the progeny with Aroona as the recurrent parent will enable the effect of these subunits on dough quality to be evaluated and compared to the other glutenin subunits present in the Aroona glutenin allele set (Chapter 4). The identification of individual or subunit pairs and the assignment of allelic designations in this study should facilitate the selection of desirable *T. dicoccoides* alleles in subsequent programs.

Crosses have been produced between well adapted Australian germplasm, including Aroona, and the derived synthetic hexaploids with the highest %UPP, AUS 27767 and AUS 27724. Further investigation of *T. tauschii* accessions is also warranted, particularly those suspected to over-express *Glu-D1* ^{t} subunits, AUS 24155 and AUS 24167. An efficient way to introgress and assess the bread making potential of the glutenin subunits associated with accessions identified in this study would be to produce synthetic hexaploid lines using these *T. tauschii* accessions and selected *T. dicoccoides* accessions.

CHAPTER 8

GENERAL DISCUSSION

Grain quality of wheat is determined by a complex matrix of variables, many of which are interdependent. The genes which determine hardness, protein composition, starch properties and enzyme and lipid composition all affect quality parameters, such as milling yield, water absorption, pasting properties, rheological properties and colour stability. The genes largely responsible for dough rheology are those which control glutenin composition and protein content. Although wheat varieties differ in their ability to accumulate protein, the variation in protein content due to genotype is small compared to the effect of growing conditions. In contrast, the alleles which determine glutenin composition explain a large proportion of the genetic variation associated with differences in end product suitability. The work reported here investigated how information regarding glutenin genotype, HMW-GS expression levels and specific protein fractions, such as %UPP, could be used to predict or could be altered to improve dough quality with a particular emphasis on bread making potential.

The effect of numerous glutenin alleles and different protein levels, in an Aroona background, on dough rheological parameters was determined. Aroona is a South Australian cultivar with only moderate dough strength which makes it an ideal background to investigate both the positive and negative effects of glutenin alleles. The HMW-GS and LMW-GS alleles that were introgressed to generate the Aroona recombinant inbred lines include those commonly found in Australian bread wheat cultivars, rare alleles not typically present and two previously undescribed alleles, *Glu-B3m* and *Glu-D3a-Gli-D1*$^{-}$.

By conducting physical dough quality tests on grain from field trials and statistical analysis of the data, it was confirmed that seasonal factors had a large impact on R_{max}, Ext. and

Area. Also, foliar application of N after anthesis significantly increased FP in all years and in turn explained 50% of the total variation associated with Ext. While glutenin alleles had little effect on Ext. (8.3%) they were the source of 75.5% and 37.8% of the variation, not accounted for by seasonal or treatment factors, related to R_{max} and Area, respectively (Chapter 4, Table 4-1).

It could be argued that increasing extensibility is not worth pursuing from a breeding perspective if such little genetic gains are to be achieved. Admittedly, in the present study, the variation (2.6% of the total variation) in extensibility resulting from the presence of different glutenin alleles was small. However, some significant differences were obtained with the predicted extensibility values ranging between 13.6 cm (*Glu-A3e*) to 15.5 cm (*Glu-B3i*). In practical terms an increase in extensibility of 1.9 cm is important in getting a new variety classified for release and suggests that allelic variation can provide useful genetic improvement in extensibility. In Australian germplasm the frequency of *Glu-D1d* is high while the frequency of *Glu-B1al* is increasing. These glutenin alleles tend to convey high dough strength, especially when combined, hence glutenin alleles which positively contribute to Ext. without causing detrimental effects to R_{max} are required. Breeding programs could combine the alleles conferring high extensibility with the alleles associated with a higher R_{max} to ensure that the final glutenin composition of a variety is likely to produce a balanced dough with moderate to high R_{max} and high extensibility. Results from the Aroona isoline trials indicate that *Glu-B3i* should be considered for this purpose and could be expected to increase Ext. by 1.4 cm or 1.5 cm at 9.4% FP when replacing common alleles such as *Glu-B3b* and *Glu-B3h*, respectively (Table 4-3, Chapter 4).

REML estimates for glutenin allele main effects (without interaction) based on the analysis of isoline quality data predicted effects ranging from -56 BU (*Glu-B3c*) to +75 BU (*Glu-*

D1d) for R_{max} with the *Glu-B1i*, *Glu-D1d*, *Glu-B3g* and *Glu-D3a-Gli-D1*$^{-}$ alleles conveying a higher maximum resistance (R_{max}) than the other alleles included in this study (Chapter 4, Table 4-3). The difference in glutenin allele main effect predictions for dough strength was 30.1 cm^2 ranging from -15.5 cm^2 (*Glu-B3c*) to +14.6 cm^2 (*Glu-D1d*) (Chapter 4, Table 4-2 and Table 4-3). In addition to the alleles associated with high R_{max}, *Glu-A1a*, *Glu-A3b*, *Glu-A3d*, *Glu-B3b*, *Glu-B3d*, *Glu-B3m* and *Glu-D3f* all had a positive influence on dough strength (Area) so that REML estimates of their main effects were not significantly different from that of *Glu-D1d* (+14.6 cm^2) which was shown to exert the greatest positive effect on Area.

Keeping in mind that the standard errors in this study were quite large, no significant glutenin gene epistaic interactions were observed for either R_{max}, Ext. or Area in this data set. Although, the differences in the effects associated with two DA isolines, DA 7+8 B3d and DA B3d D3a, and the effects predicted based on individual additive main effects of the substituted alleles were consistently high and approaching significance for R_{max}, Area and to a lesser extent Ext. (Chapter 4, Table 4-4, Table 4-6 and Table 4-5, respectively). Interestingly both these DA isolines contained the *Glu-B3d* allele and the putative epistatic effects were positive for DA 7+8 B3d and negative for DA B3d D3a.

Any speculation regarding epistatic interactions involving *Glu-B3d* requires further investigation, ideally with a more balanced data set and smaller standard errors and confirmation of the glutenin alleles present should be obtained using SDS-PAGE analysis of the actual flour samples (one from each year) used for the dough rheological tests. This has been done for DA 7+8, B3d (data not shown) as there was evidence to support the possibility of a significant positive epistatic interaction between *Glu-B1b* and *Glu-B3d* at higher protein levels. The ANOVA estimates for isolines where the years were analysed independently (Appendix II, Table 5) show that the single allele substitution of *Glu-B3d*

(Aril 24-3) resulted in a decrease in R_{max} in all years (significant in 2002 and 2003 at the $P<0.05$ level) whereas substituting *Glu-B1c* with *Glu-B1b* (Aril 6-4 and Aril 7-1) resulted in predicted R_{max} values similar to Aroona. The double substitution of alleles present in DA 7+8 B3d tended to increase R_{max} but this increase was highly significant ($P<0.001$) only at higher protein levels (2001, FP = 9.47%; 2002 FP = 11.45%).

A similar effect was found for Ext. where predictions for the single allele substitutions did not significantly differ from Aroona, except for the decrease in Ext. for *Glu-B1b* in 2003 ($P<0.05$) level. Whereas DA 7+8 B3d increased extensibility by ≈ 2.5 cm ($P<0.001$) in 2002 (FP = 11.45%) and by ≈ 0.9 cm in 2003 (FP = 8.52%) (Appendix II, Table 6). Again for Area, the isolines Aril 24-3 (*Glu-B3d*) and Aril 6-4 and Aril 7-1 (*Glu-B1b*) tended to decrease Area but the DA 7+8 B3d isoline significantly increased Area by ≈ 17 cm^2 in 2001 ($P<0.01$; FP = 9.47%) and by ≈ 28 cm^2 in 2002 ($P<0.001$; FP = 11.45%) relative to the Area prediction for Aroona (Appendix II, Table 7).

Clearly, as a consequence of this work, some interesting points need further investigation to clarify whether:

1) there is a significant positive epistatic interaction between *Glu-B1b* and *Glu-B3d*;
2) the REML R_{max}, Ext. and Area predictions for *Glu-B3d* are biased because of the possible positive epistatic effect observed for DA 7+8 B3d. If this is the case, REML predictions for *Glu-B3d* main effects could be overestimated (DA 7+8, B3d was present in all four years while DA B3d, D3a was only present in two of the years);
3) *Glu-B3d* only conveys a positive effect on Ext. at higher protein levels when combined with *Glu-B1b* or is there also a positive epistatic interaction between

Glu-B3d and *Glu-B1u* (7*+8), a more common allele in Australian germplasm than *Glu-B1b*;

4) *Glu-B3d* would interact with *Glu-B1al* to increase Ext. at higher protein levels since the same 1Bx7 subunit is present but over-expressed;
5) the interaction would still exist if *Glu-B1b* and *Glu-B3d* were combined with alleles other than those present in Aroona eg. *Glu-D1d* (5+10) instead of *Glu-D1a* (2+12);
6) a similar epistatic interaction occurs between *Glu-B1b* and *Glu-B3i*; and
7) the development of new DA substitution lines involving different combinations of the glutenin alleles present in this study would identify other epistatic interactions between glutenin alleles.

Table 8-1 shows comparisons between examples of the highest and lowest glutenin allele combinations for R_{max}, Area and Ext. Predictions are based on assuming additive effects of the alleles for R_{max}, Area and Ext. relative to the means for all alleles calculated by REML. For R_{max} the base was 176 BU while for Ext. and Area the base figures were 14.4 cm and 48.7 cm^2, respectively. Low R_{max} and Area were associated with the same allele combinations and *Glu-A1c*, *Glu-A3e* and *Glu-D3b* were also present in the combination associated with low Ext. So these alleles are not considered to be desirable and, with the exception of *Glu-D3b*, their frequency is low in Australian germplasm (Eagles *et al.*, 2006). Similarly, the same allele combination was associated with higher values for both R_{max} and Area (*Glu-A1a*, *Glu-B1i*, *Glu-D1d*, *Glu-A3d*, *Glu-B3g* and *Glu-D3a-Gli-D1*$^-$) with *Glu-A1a* and *Glu-B1i* also contributing to increase Ext (Table 8-1). The predictions shown in Table 8-1 clearly show the extent that dough rheological parameters can be influenced by the additive nature of glutenin alleles and why the correct choice of combination is imperative. The highest predictions for R_{max} and Area was 418 BU and 109.7 cm^2, respectively, a significant increase from the baseline values of 176 BU and

		Glu-A1	*Glu-B1*	*Glu-D1*	*Glu-A3*	*Glu-B3*	*Glu-D3*	Additive allele effect
R_{max}	(high)	*a*	*i*	*d*	*d*	*g*	*a-Gli-D1*$^-$	= 418 BU
	(low)	*c*	*a*	*f*	*e*	*c*	*b*	= 64 BU
Area	(high)	*a*	*i*	*d*	*d*	*g*	*a-Gli-D1*$^-$	= 109.7 cm^2
	(low)	*c*	*a*	*f*	*e*	*c*	*b*	= −16.7 cm^2
Ext.	(high)	*a*	*i*	*a*	*a*	*i*	*d*	= 17.2 cm
	(low)	*c*	*d*	*d*	*e*	*a*	*b*	= 11.2 cm

Table 8-1 Predictions for glutenin allele combinations based on REML estimates for additive allele main effects without interaction (Table 4.2, Chapter 4) that would produce doughs with the highest and lowest R_{max}, Area and Ext. at a FP of 9.4%. Results are relative to the means for all alleles as calculated by REML (R_{max} = 176 BU; Area = 48.7 cm^2; Ext. = 14.4 cm).

48.7cm^2. It would be interesting to produce an Aroona isoline with the allele combination *Glu-A1a* (1), *Glu-B1i* (17+18), *Glu-D1d* (5+10); *Glu-A3d*, *Glu-B3g* and *Glu-D3a-Gli-D1*$^-$ and compare the results for dough rheology with the R_{max} and Area predictions reported here. The negative result obtained for Area by the allele combination associated with low dough strength is impossible as it assumes only additive effects but illustrates that flour derived from varieties containing these glutenin alleles would not be suitable for bread manufacture. In fact, the low Ext. due to the presence of *Glu-A1c* (null), *Glu-D1d* (5+10), *Glu-A3e* (null) and *Glu-D3b* would result in a dough unsuitable for any end-use. For Ext., a highly significant genetic effect was apparent with predictions ranging 11.2 cm to 17.2 cm. Again, comparisons between an actual isoline that contains *Glu-A1a*, *Glu-B1i*, *Glu-D1a*; *Glu-A3a*, *Glu-B3i* and *Glu-D3d* and Aroona would be very interesting.

Recently the question was asked "How would you use this information to improve the dough quality of the widely grown South Australian variety, Yitpi?". Yitpi has the glutenin genotype *Glu-A1a* (1), *Glu-B1u* (7*+8), *Glu-D1d* (5+10); *Glu-A3c*, *Glu-B3h* and *Glu-D3c* and is considered to have good dough strength and moderate extensibility

(Mosionek, pers. comm.). Based on the effects reported here the R_{max} and Ext. predictions for Yitpi would be (assuming the effects of *Glu-B1b* and *Glu-B1u* are not significantly different) :

	Base	*Glu-A1a*	*Glu-B1u*	*Glu-D1d*	*Glu-A3c*	*Glu-B3h*	*Glu-D3c*	prediction
R_{max}	176 BU	+ 13	+ 16	+ 75	+ 2	– 7	– 2	= 273 BU
Ext.	14.4 cm	+ 0.4	0	– 0.4	+ 0.2	– 0.4	– 0.2	= 14.0 cm

Interestingly, the HMW-GSs in Yitpi do not contribute to Ext. however, the alleles that should be changed are those which exert a negative effect on both R_{max} and Ext. With minimal genetic manipulation, this could be achieved by substituting *Glu-B3h* with *Glu-B3g* and *Glu-D3c* with *Glu-D3a-Gli-D1*$^{-}$. R_{max} and Ext. predictions based on the additive effects of these substitutions would be :

	Base	*Glu-A1a*	*Glu-B1u*	*Glu-D1d*	*Glu-A3c*	*Glu-B3g*	*Glu-D3a-Gli-D1*$^{-}$	prediction
R_{max}	176 BU	+ 13	+ 16	+ 75	+ 2	+ 46	+ 41	= 369 BU
Ext.	14.4 cm	+ 0.4	0	– 0.4	+ 0.2	+ 0.4	+ 0.3	= 15.3 cm

If lower R_{max} and greater Ext. was desired, instead of substituting *Glu-B3h* with *Glu-B3g*, *Glu-B3i* could be used. Yitpi would then be predicted to have an R_{max} of 316 BU and an Ext. of 16 cm.

The similarity in REML predictions that rank the effects of glutenin alleles on R_{max} and Area obtained here is not surprising, particularly when the protein content of the samples is considered. The mean FP in this study was 9.4% although the raw NIR FP data (not shown) for individual samples ranged from 6% to 15.6% over the four years. The rheological data obtained showed that at low protein, dough strength (Area) correlates very highly and positively with R_{max}, while at higher protein levels Area correlates highly and

significantly with both R_{max} and Ext., reflecting the balance between these two important quality parameters (Figure 4-3, Chapter 4). The variable FP of samples in this study could have adversely affected the reliability of results reported here. FP and Ext. in low protein years were less positively correlated than in years where higher protein levels were achieved. Either the relationship between FP and Ext. is not linear or errors occurred, which were noted at the time, when measuring extensograph Ext. with a single pull of a 75g dough piece low in protein content. This suggests that for more meaningful research into the relationship between glutenin alleles and bread making quality, especially for Ext. where the genetic effects are small compared to environmental factors, the samples should be analysed in duplicate using a larger dough piece (150g) or possess a flour protein content considered suitable for the manufacture of pan-bread (>10% FP). In addition, Ext. results are also dependant on the dough's developmental stage when tested. The optimum mixing time for Ext. measurement corresponds with peak dough development when the gluten matrix has been fully developed. At this stage, the dough surface takes on a sheen and is amenable to stretching (MacRitchie, 1992). Unfortunately, extensograph data is routinely obtained using a pre-set mixing time (usually 5 min for Quadrumat Junior milled flours), from a research perspective it would be useful to conduct further experiments that allow extensograph tests to be conducted on samples that have been mixed to peak dough development time as determined by the farinograph.

A glutenin allele (*Glu-B1al*) has been identified and shown to convey exceptionally high dough quality properties known to be positively correlated with bread making potential. Cultivars that contain the *Glu-B1al* allele produce very strong and extensible doughs. Typically, the presence of the *Glu-B1al* (7+8*) allele is associated with the over-expression of HMW-GS 1Bx7 (Marchylo *et al.*, 1992; Gianibelli *et al.*, 2002). Since this allele was not present in the Aroona isoline glutenin allele set, no rheological data could be reported here. Therefore investigations of the *Glu-B1al* allele were directed at finding the

origin of this allele and to identify cultivars in which it is present. To do this a RP-HPLC protocol, based on previously published methods was implemented and used in combination with pedigree data to study wheat varieties released in a number of countries. The *Glu-B1al* allele in the varieties reported here, including the Australian cultivars Kukri, Chara and the breeding line CD 87, could be traced, at least through one parent, to the Argentinian bread wheat cultivar Klein Universal II (Figure 5-3, Chapter 5).

The frequency of the *Glu-B1al* allele in Australian germplasm is increasing and concerns have been raised regarding the impact this may have. In contrast with export market demands, the domestic market does not support efforts to increase dough strength because of the increased work input required to mix stronger doughs. However, the development of varieties with over-strong dough properties would depend largely on the other glutenin alleles present. It has been shown that the *Glu-B1al* allele in combination with *Glu-D1a* (2+12) has a shorter mixing time, a higher extensibility and no adverse effect on dough stability, compared to when it is combined with *Glu-D1d* (5+10) (Tonkin and Cornish, 2004). In fact, the mixing requirement (arrival time) for the *Glu-B1al* and *Glu-D1a* combination was not significantly different from *Glu-B1u* (7*+8) combined with *Glu-D1d*. Alternatively, Australia could follow the Canadian example where a separate market classification, Canada Western Extra Strong (CWES), based on the variety Glenlea (*Glu-B1al* and *Glu-D1d*), has been established to reflect the demand for wheats that produce extra strong doughs. CWES wheats are reported to be ideal for blending purposes, the production of frozen dough products that maintain baking quality after thawing, the 'sponge and dough' method of bread baking and the production of salt-reduced bread products (Cereal Research Centre, Agriculture and Agri-Food Canada, 2003; Butow *et al.*, 2002).

RP-HPLC also provided the opportunity to investigate the expression levels of numerous

HMW-GS alleles and their relationship with dough quality. It was shown that for the majority of hexaploid cultivars, the B-genome contributed the highest percentage of HMW-GSs. The only exception in this study were varieties that contained *Glu-B1d* (6+8*), where the D-genome contributed the most. Cultivars that possessed the *Glu-B1al* allele contained a significantly higher ($P<0.001$) proportion of HMW-GSs (56.80 ± 3.25 %) encoded by the B-genome compared to the expression levels of the A- and D-genome subunits (Table 5-1, Chapter 5). In addition, the mean ratio of *Glu-B1*x subunits to total x-type HMW-GSs in cultivars which possess the *Glu-B1al* allele (0.58) was significantly higher ($P<0.001$) than for those having other *Glu-B1* alleles. Statistical analysis of cultivars with the *Glu-B1* alleles *i* (0.45), *f* (0.45), *c* (0.41), *u* (0.43) and *ak* (0.42) showed that the Bx:Ax+Bx+Dx ratio was not significantly different ($P<0.05$) within this group, while the ratio was significantly higher ($P<0.05$) than in cultivars that possess the *Glu-B1e* (0.36) and *d* (0.29) alleles, with *Glu-B1e* and *Glu-B1d* were not significantly different from each other. This division of *Glu-B1* alleles into three sub-groups (Group 1: *Glu-B1al*; Group 2: *Glu-B1i*, *f*, *c*, *u* and *ak*; and Group 3: *Glu-B1e* and *d*) reflects their known influence on dough strength, which is extra strong, strong-to-medium and weak, respectively (Table 5-2, Chapter 5). This suggests that the overall proportion of *Glu-B1* subunits, relative to the total amount of HMW-GSs expressed, has a major influence on dough strength and that the proportion of 1Bx to other x-type subunits, as determined by RP-HPLC, could also be used to predict dough quality.

A possible explanation of why *Glu-B1* x-type subunits have a greater influence on dough quality than other x-type subunits could be related to differences in amino acid sequences that cause structural/conformational changes, enabling them to participate more effectively in the formation of polymeric protein. 1Bx subunits are the smallest x-type subunits and although the positions of the first and last cysteine residues are conserved in 1Ax, 1Bx and 1Dx subunits, the position of the central two cysteines is not (positions 43 and 58, 38 and

53, 46 and 61 for 1Ax, 1Bx and 1Dx subunits, respectively) (Ikeda *et al.*, 2002). Another possible mechanism relates to the expression levels of *Glu-B1* x-type HMW-GSs which were shown to be greater in varieties associated with moderate to very high dough strength (Table 5-2, Chapter 5). Reconstitution studies have shown that x-type HMW-GSs contribute more to R_{max} then y-type subunits (Wieser et al., 1994) so the resulting increase in the x-:y-type HMW-GS ratio due to the over-expression of 1Bx7 could explain the high dough strength associated with the presence of the *Glu-B1al* allele.

In certain circumstances it is paramount that breeders can conduct selections in early generations, particularly when introducing exotic genetic material (ie. 'muddying' their gene pool), a practice that is essential if significant advances in cultivar development is to be achieved. Similarly, since the market demand for wheat requires that varieties with different dough qualities are developed, it is also important that there are rapid small-scale tests available that can be used to reliably predict end product suitability. In accordance with what has previously been published, the results here showed that the relative quantity of polymeric protein fractions, as determined by SE-HPLC, can be used to predict dough strength. Correlations between SE-HPLC %UPP and Ext., R_{max}, Area, FDDT and FDS were positive and highly significant. Also, %UPP was found to be significantly and negatively correlated with FDBD, a measure of dough weakness (Chapter 6, Table 6-1).

Although the use of SE-HPLC to determine quantities of specific protein fractions is relatively simple, it is time consuming and requires the use of expensive specialised equipment. Clearly, it would be advantageous to use a small-scale screening method to eliminate germplasm with undesirable protein quality traits prior to undertaking the more demanding traditional quality testing methodologies. Accordingly, this thesis described work relating to the development and validation of a simple screening method that is robust, has a high throughput relative to SE-HPLC and inexpensive. The Turbidity assay

uses the SE-HPLC protocol to solubilise protein into specific fractions, 10% TCA to precipitate the protein and spectrophotometric analysis at 450nm to determine the percentage of SDS-insoluble protein (%IP). %IP correlated highly with dough quality attributes, particularly with extensograph R_{max} and Area, farinograph FDDT and FDS and SE-HPLC %UP and %UPP making it a most useful early generation selection tool (Table 6-1, Chapter 6). The Turbidity assay was not compared with other small-scale screening methods (eg. Zeleny volume and SDS-Sedimentation volume) since some wheat breeding companies such as, Australian Grain Technologies, do not currently routinely conduct these early generation tests, not only because they are labour intensive and time consuming, but because their current breeding objectives do not require a large introgression of exotic material. Logically, the Turbidity assay would be the method of choice when breeding material does require early generation screening.

In addition to investigating glutenin alleles present in cultivated hexaploids, wild wheat relatives were screened for strong protein characteristics, namely for high %UPP and %IP, using SE-HPLC and the Turbidity assay, respectively. SDS-PAGE (uniform and gradient) was conducted to assess the glutenin genetic variability and characterise previously undescribed HMW-GS alleles that have the ability to interact and form polymeric protein. *T. dicoccoides* accessions were studied to investigate *Glu-1* variation associated with progenitor A- and B-genomes. Extensive allelic variation in glutenin subunit composition was evident and allelic designations were assigned to individual HMW-GSs or subunit pairs (Table 7-1, Chapter 7). In the future, these allelic designations should facilitate the selection of putative desirable *T. dicoccoides* HMW-GSs in subsequent generations.

SE-HPLC was also used to screen the D-genome progenitor, *T. tauschii* and its synthetic hexaploid derivatives, for protein quality attributes. There was a wide range of %UPP levels present in both *T. tauschii* (25.4-67.7%; Table 7-3, Chapter 7) and synthetic

hexaploid accessions (33.2-68.0%; Table 7-4, Chapter 7) and those with the greatest amount of %UPP were selected for introgression into Australian germplasm. Already, F_1 progeny derived from crosses between *T. dicoccoides*, synthetic hexaploids and well-adapted Australian bread wheat lines have been produced. Clearly, to evaluate the potential ecomomic value of the new glutenin subunits described in this thesis, a backcrossing program and quality tests on the hexaploid progeny need to be conducted to determine their effects on dough rheology and bread making quality.

Changing world wheat export and import patterns presents both opportunities and challenges for the Australian wheat industry. With the emergence of significant "non-traditional exporters" of relatively undifferentiated quality grain entering the international market, competition for the higher value markets is likely to increase considerably. In order to remain competitive in the international marketplace, Australia needs to supply high quality grain suited to specific end-uses. The results presented in this thesis describe the relationship between numerous glutenin alleles and some of the functional properties of wheat flour dough and illustrated that alleles present at each of the *Glu-1* and *Glu-3* loci can have a large effect on dough rheology and marketability. Meanwhile, the concept that new wheat varieties could be developed more efficiently by selecting both *Glu-1* and *Glu-3* alleles simultaneously was confirmed. Never before has the effect of such a comprehensive set of glutenin alleles on dough rheology been investigated in a common background and although this study served to augment previous studies based on fewer alleles, the effect of many of the less well characterised alleles, particularly LMW-GS alleles was shown.

In the short term, increasing the frequency of glutenin alleles associated with strength and extensibility reported here, providing the right balance between dough strength and extensibility is achieved, would significantly improve the bread making potential of

Australian wheat varieties. Longer term goals for specific end products, such as frozen doughs, would be the development of elite quality germplasm through the introgression of new glutenin alleles identified in accessions of undomesticated relatives of wheat.

The work reported in this thesis regarding the range of genetic variation in endosperm storage proteins associated with the *Glu-1* loci in wheat progenitors and wheat relatives is by no means exhaustive. The search or 'mining' of potential new glutenin alleles in under-exploited wheat species, including both HMW-GSs and LMW-GSs, that may lead to significant improvements in dough quality is important and should be ongoing. Support received for this project from industry partners and research organisations is testimony to this.

CHAPTER 9

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CHAPTER 10

APPENDICES

Appendix I

SDS-PAGE of Aroona isolines .....

Appendix II

Isoline response to treatment .....

Appendix III

Glutenin allele interaction tables .....

Appendix IV

Vawser M-J and Cornish G B (2004) AJAR 55, 577-588 .....

Appendix V

SDS-PAGE of *T. dicoccoides* .....

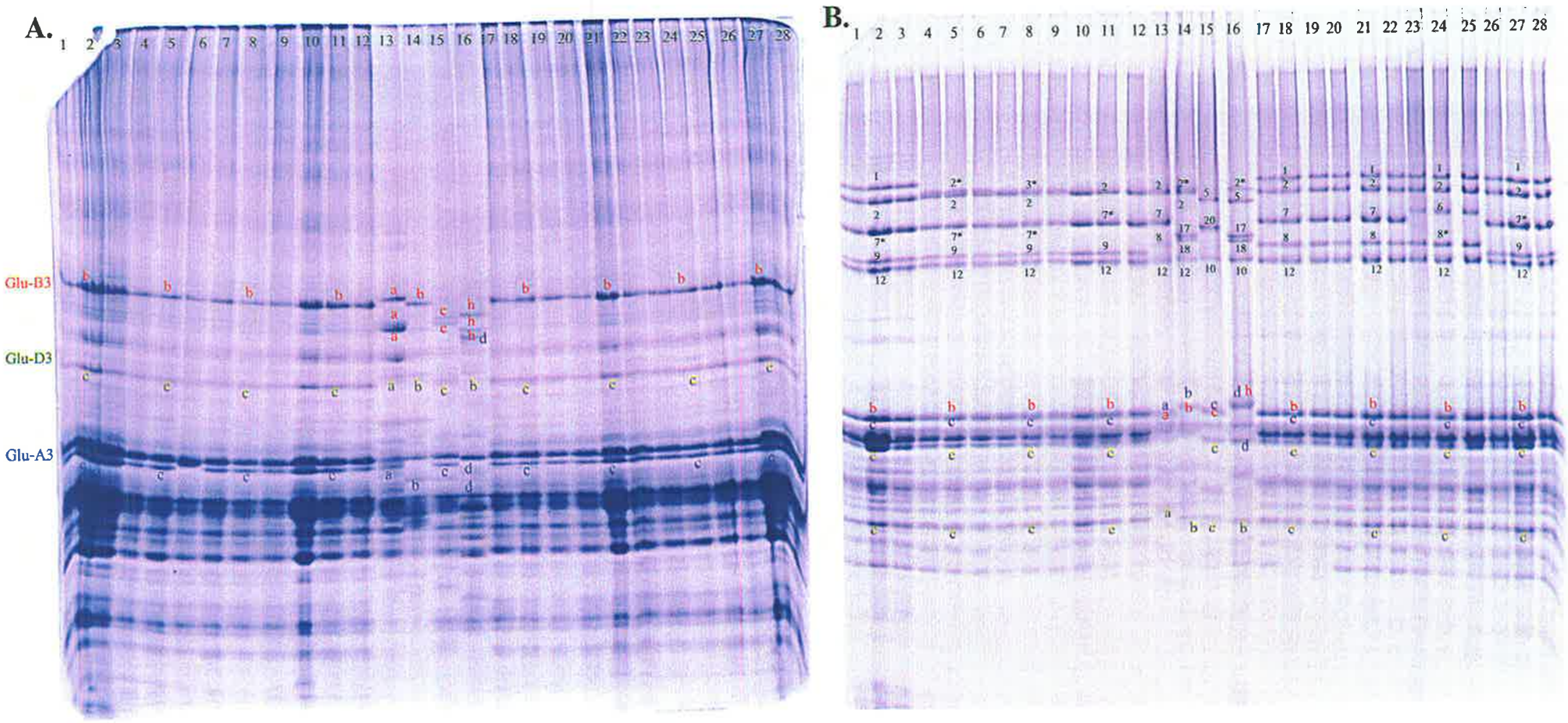

Figure 1. SDS Gradient PAGE (8-12%) of Aroona Isolines **A.** Gliadins and **B.** Glutenins. Lanes 1-3, Aroona; Lanes 4-6, Aril 2-4; Lanes 7-9, Aril 4-3; Lanes 10 -12, Aril 3-2; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne ; Lanes 17-19, Aril 7-1; Lanes 20-22, Aril 6-4; Lanes 23-25, Aril 9-3; Lanes 26-28, Aroona.

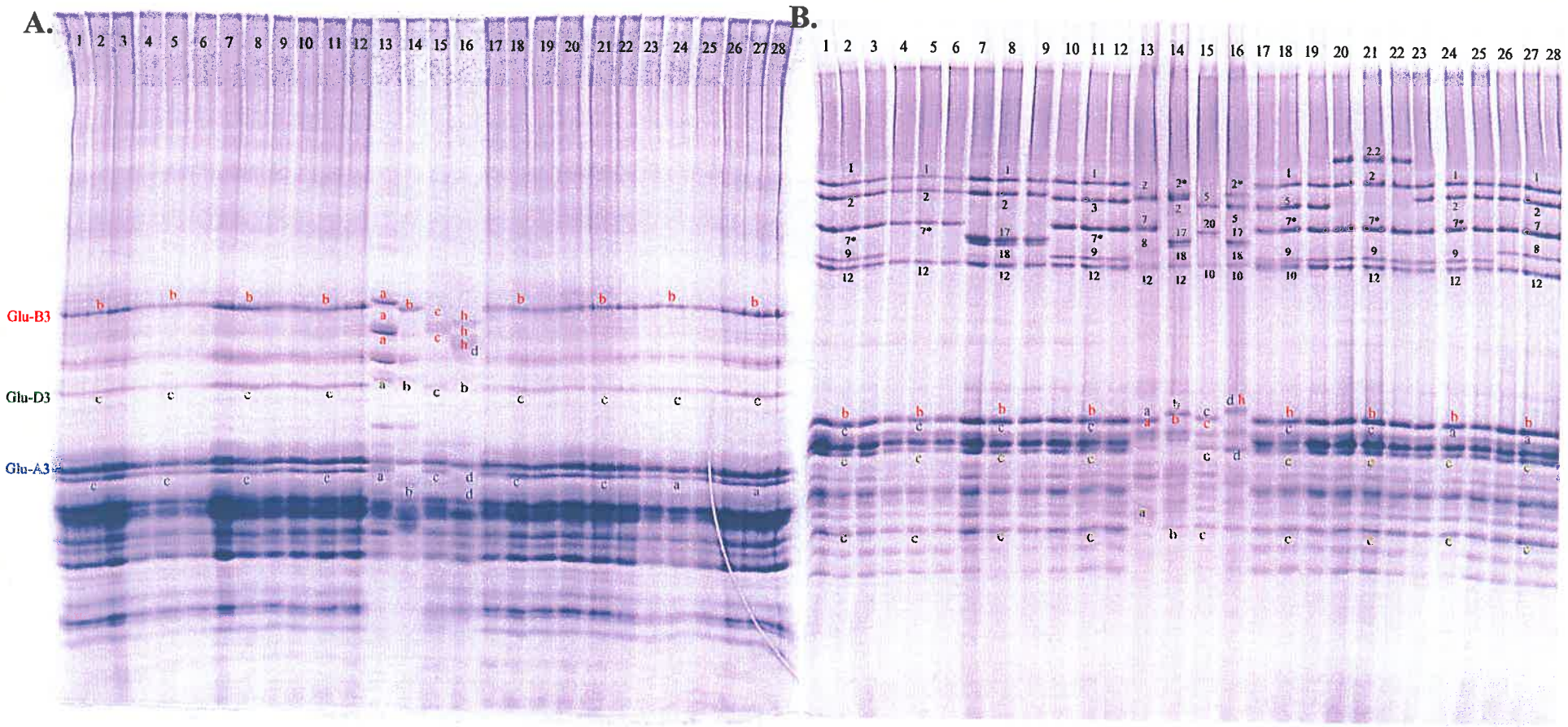

Figure 2. SDS Gradient PAGE (8-12%) of Aroona Isolines **A.** Gliadins and **B.** Glutenins. Lanes 1-3, Aroona; Lanes 4-6, Aril 5-2; Lanes 7-9, Aril 10-1; Lanes 10 -12, Aril 12-3; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-19, Aril 13-3; Lanes 20-22, Aril 14-3; Lanes 23-25, Aril 15-4; Lanes 26-28, DA 7+8 A3a.

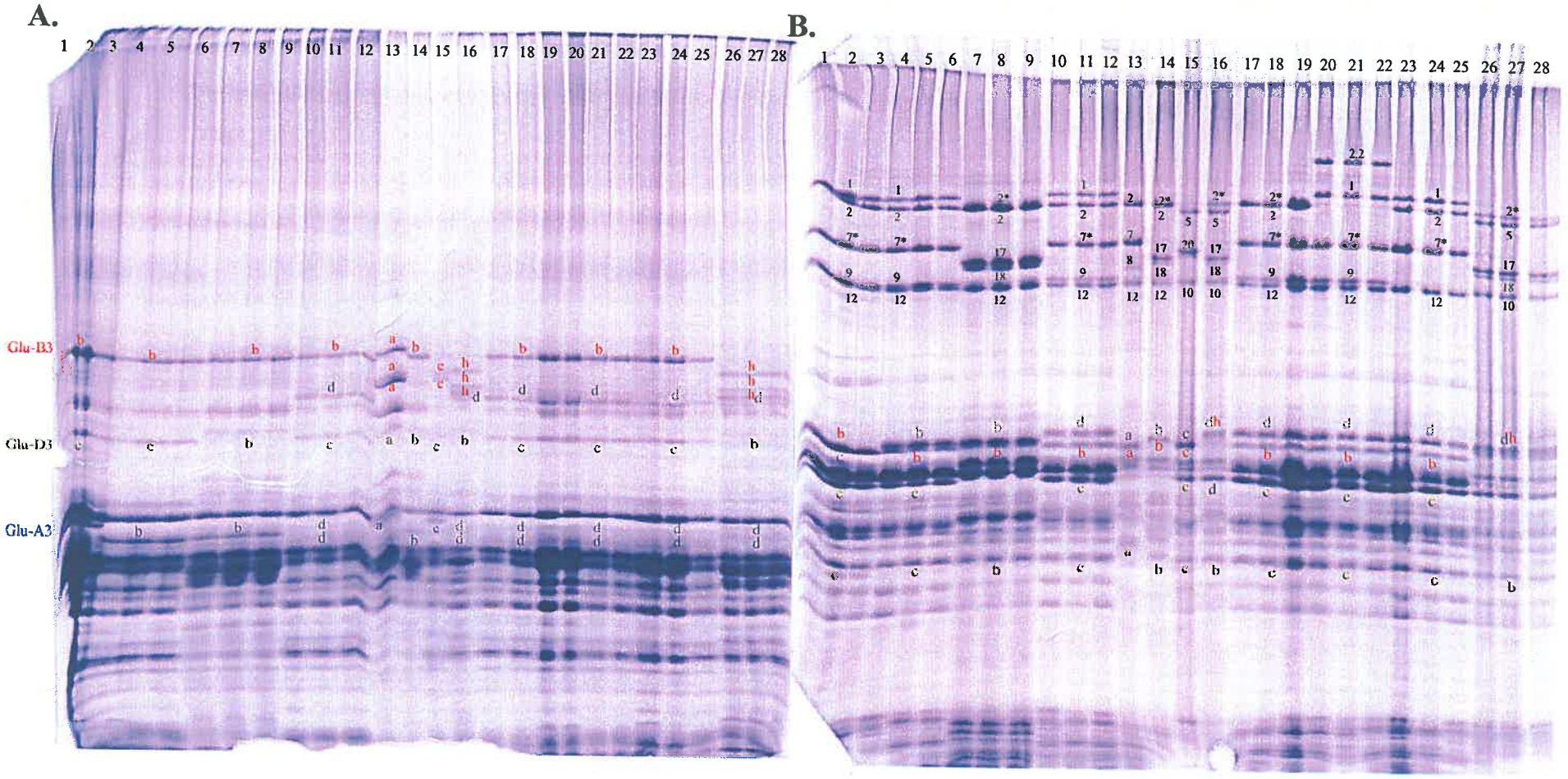

Figure 3. SDS Gradient PAGE (8-12%) of Aroona Isolines **A.** Gliadins and **B.** Glutenins. Lanes 1-3, Aroona; Lanes 4-6, Aril 16-1; Lanes 7-9, Gabo; Lanes 10 -12, Aril 18-9; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-19, DA 2* A3d; Lanes 20-22, DA 2.2+12 A3d; Lanes 23-25, DA 7* A3d; Lanes 26-28, Wilgoyne.

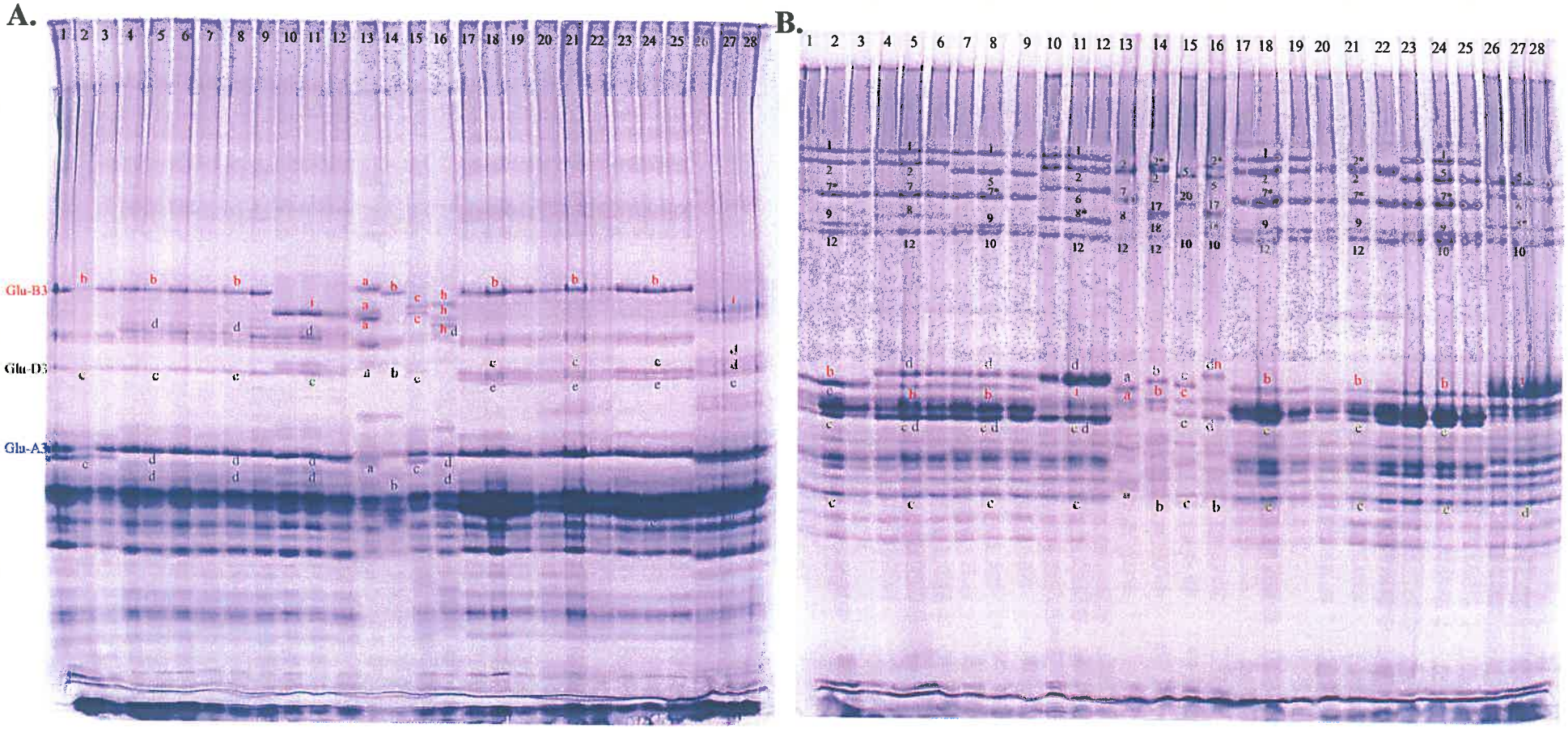

Figure 4. SDS Gradient PAGE (8-12%) of Aroona Isolines **A.** Gliadins and **B.** Glutenins. Lanes 1-3, Aroona; Lanes 4-6, DA 7*+8 A3d; Lanes 7-9, DA 5+10 A3d; Lanes 10 -12, TA 6+8* A3d B3i; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-19, Aril 19-2; Lanes 20-22, DA 2* A3e; Lanes 23-25, DA 5+10 A3e; Lanes 26-28, Jufy-1.

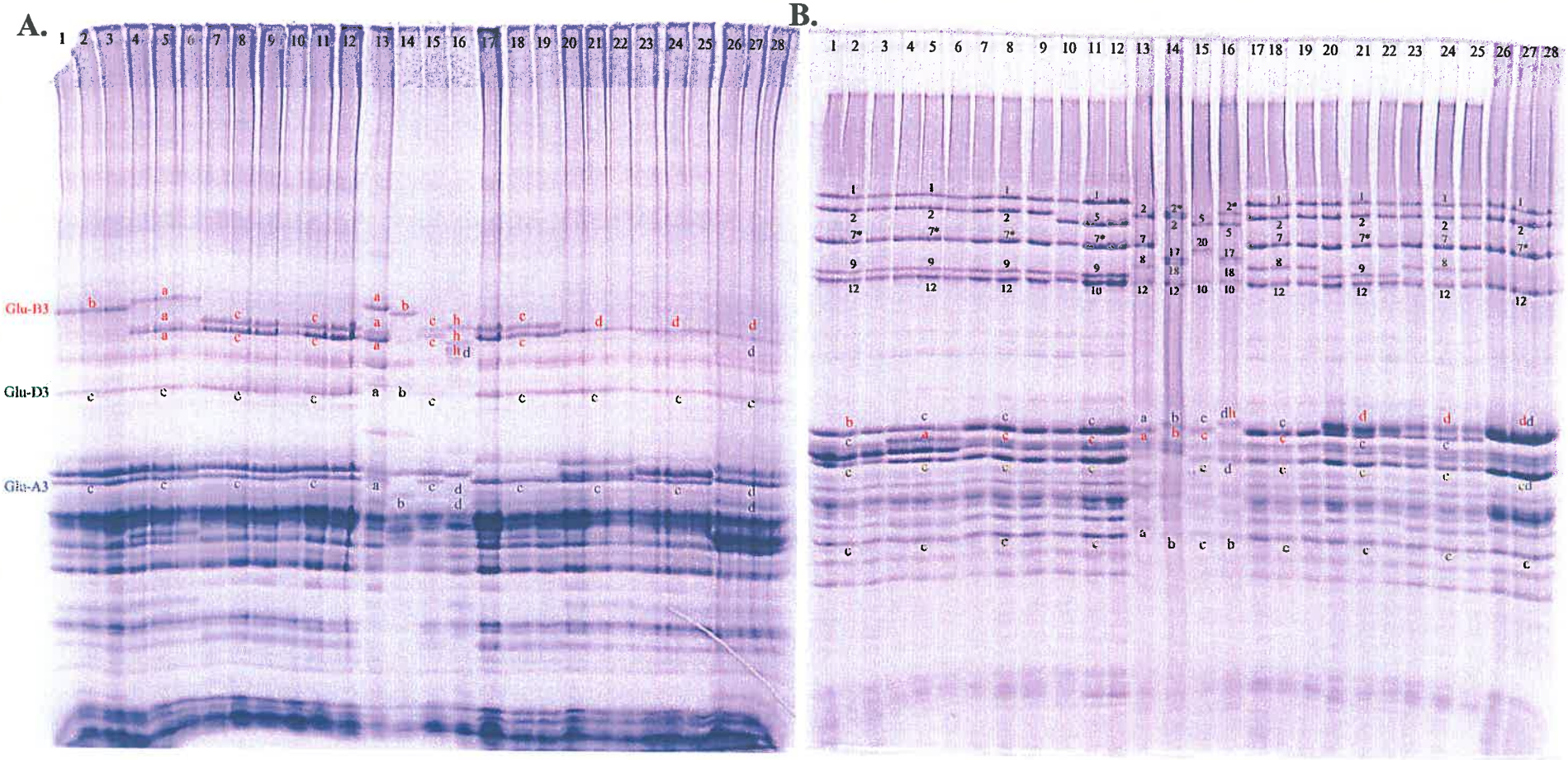

Figure 5. SDS Gradient PAGE (8-12%) of Aroona Isolines **A.** Gliadins and **B.** Glutenins. Lanes 1-3, Aroona; Lanes 4-6, Aril 21-2; Lanes 7-9, Aril 23-4; Lanes 10 -12, DA 5+10 B3c; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-19, DA 7+8 B3c; Lanes 20-22, Aril 24-3; Lanes 23-25, DA 7+8 B3d; Lanes 26-28, Orca.

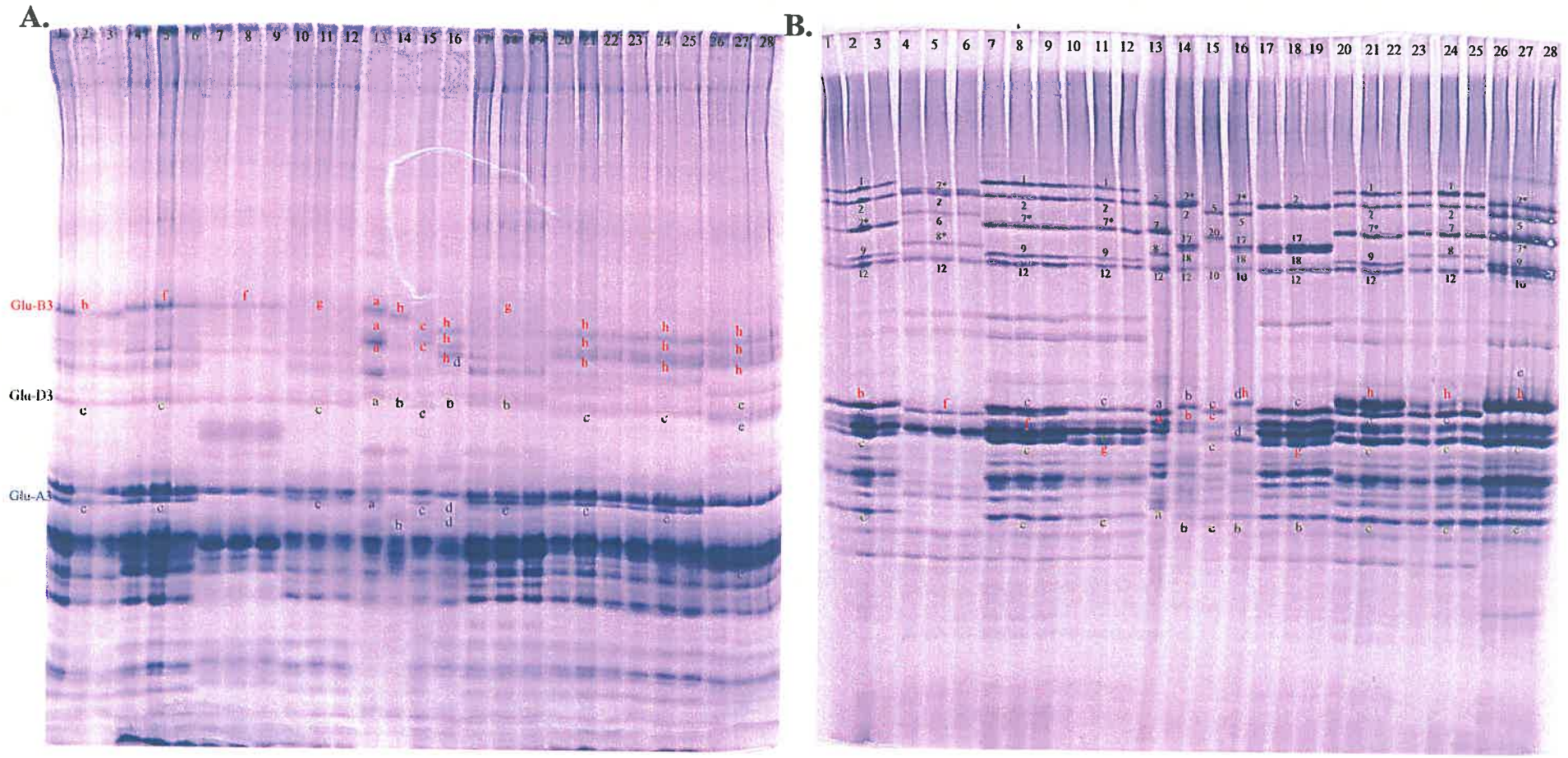

Figure 6. SDS Gradient PAGE (8-12%) of Aroona Isolines **A.** Gliadins and **B.** Glutenins. Lanes 1-3, Aroona; Lanes 4-6, Single allele B3f; Lanes 7-9, Aril 26-1; Lanes 10 -12, Aril 27-6; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-19, Millewa; Lanes 20-22, Aril 28-4; Lanes 23-25, DA 7+8 B3h; Lanes 26-28, Sonalika.

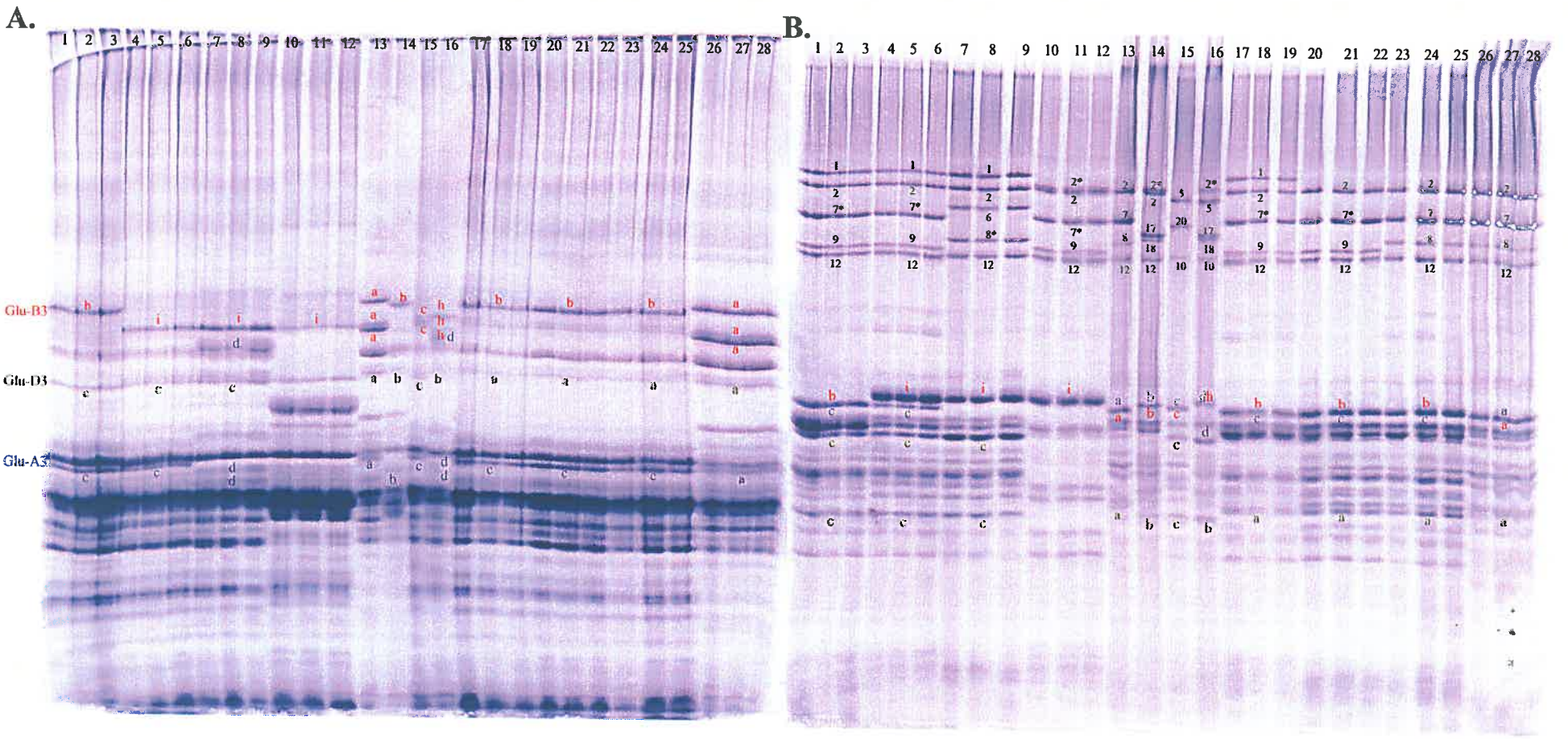

Figure 7. SDS Gradient PAGE (8-12%) of Aroona Isolines **A.** Gliadins and **B.** Glutenins. Lanes 1-3, Aroona; Lanes 4-6, Aril 29-4; Lanes 7-9, TA 6+8* A3d B3i; Lanes 10 -12, Single allele B3i; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-19, Aril 30-2; Lanes 20-22, DA null D3a; Lanes 23-25, TA null 7+8 D3a; Lanes 26-28, Chinese Spring.

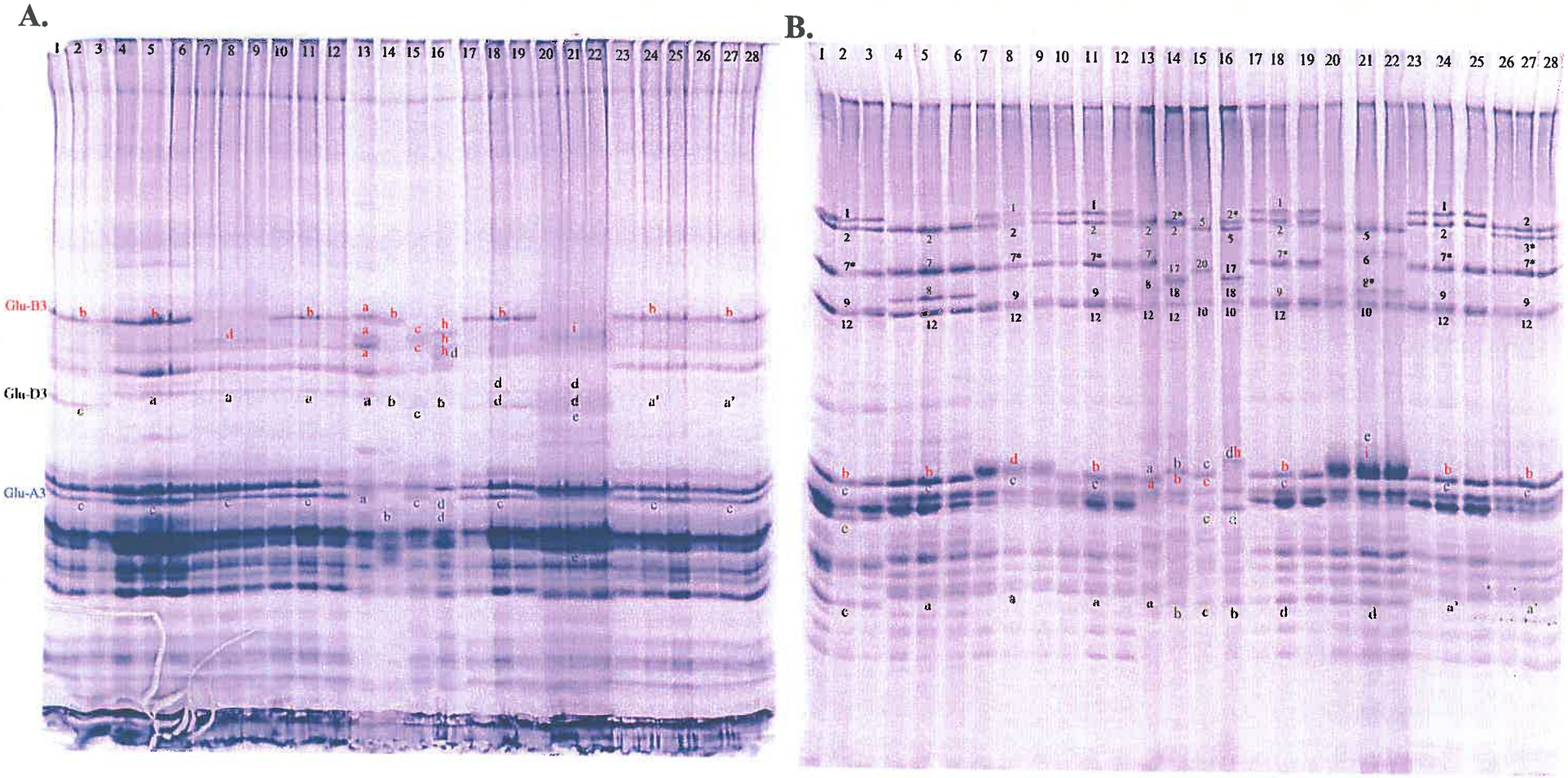

Figure 8. SDS Gradient PAGE (8-12%) of Aroona Isolines **A.** Gliadins and **B.** Glutenins. Lanes 1-3, Aroona; Lanes 4-6, TA null 7+8 D3a; Lanes 7-9, DA B3d D3a; Lanes 10 -12, Aril 31-2; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-19, Aril 33-1; Lanes 20-22, Jufy-1; Lanes 23-25, Aril 34-1; Lanes 26-28, DA 3* D3a-Gli-D1-.

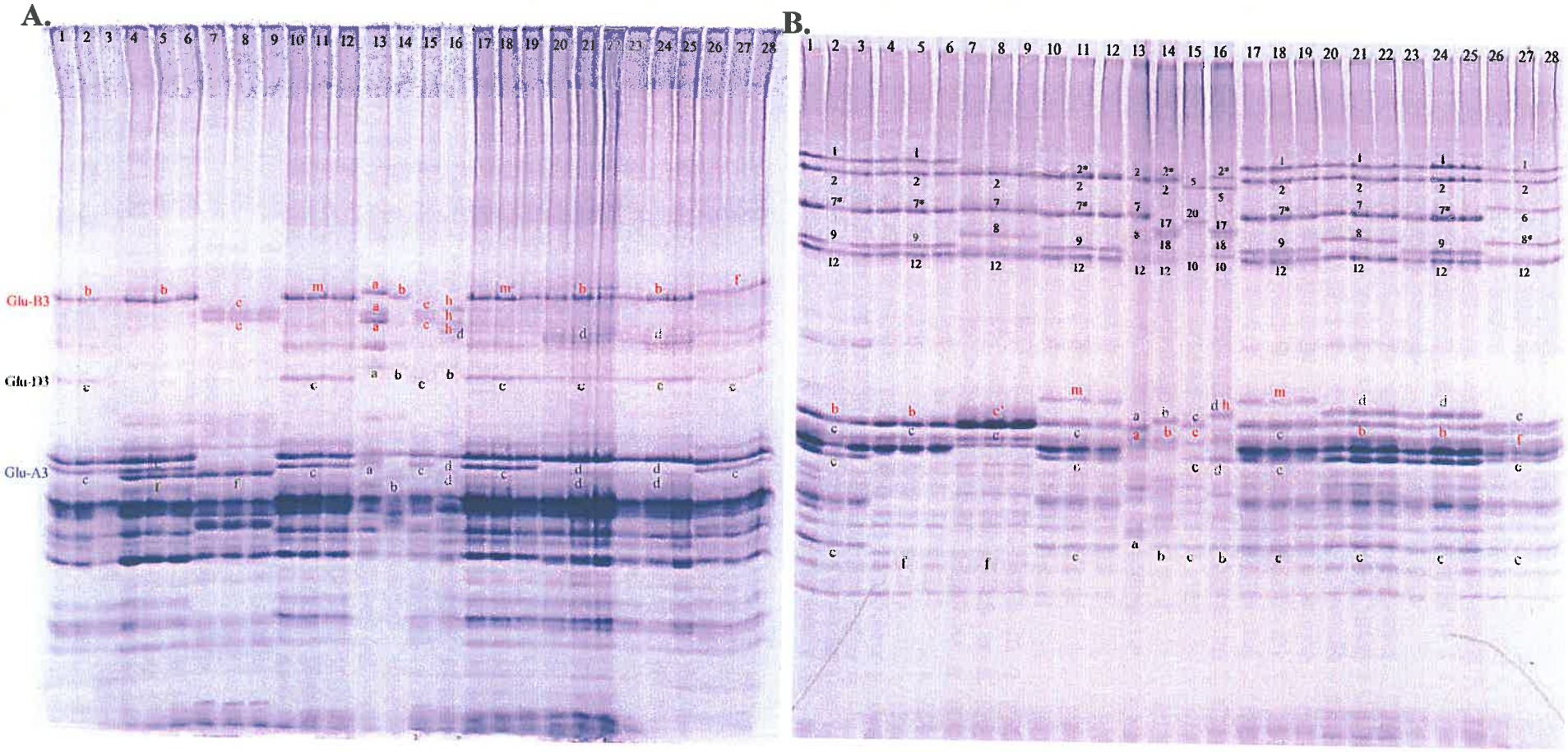

Figure 9. SDS Gradient PAGE (8-12%) of Aroona Isolines **A.** Gliadins and **B.** Glutenins. Lanes 1-3, Aroona; Lanes 4-6, Aril 35-1; Lanes 7-9, India 115; Lanes 10 -12, DA 2* B3m; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-19, Aril 27-3; Lanes 20-22, DA 7+8 A3d; Lanes 23-25, Aril 18-5; Lanes 26-28, DA 6+8* B3f.

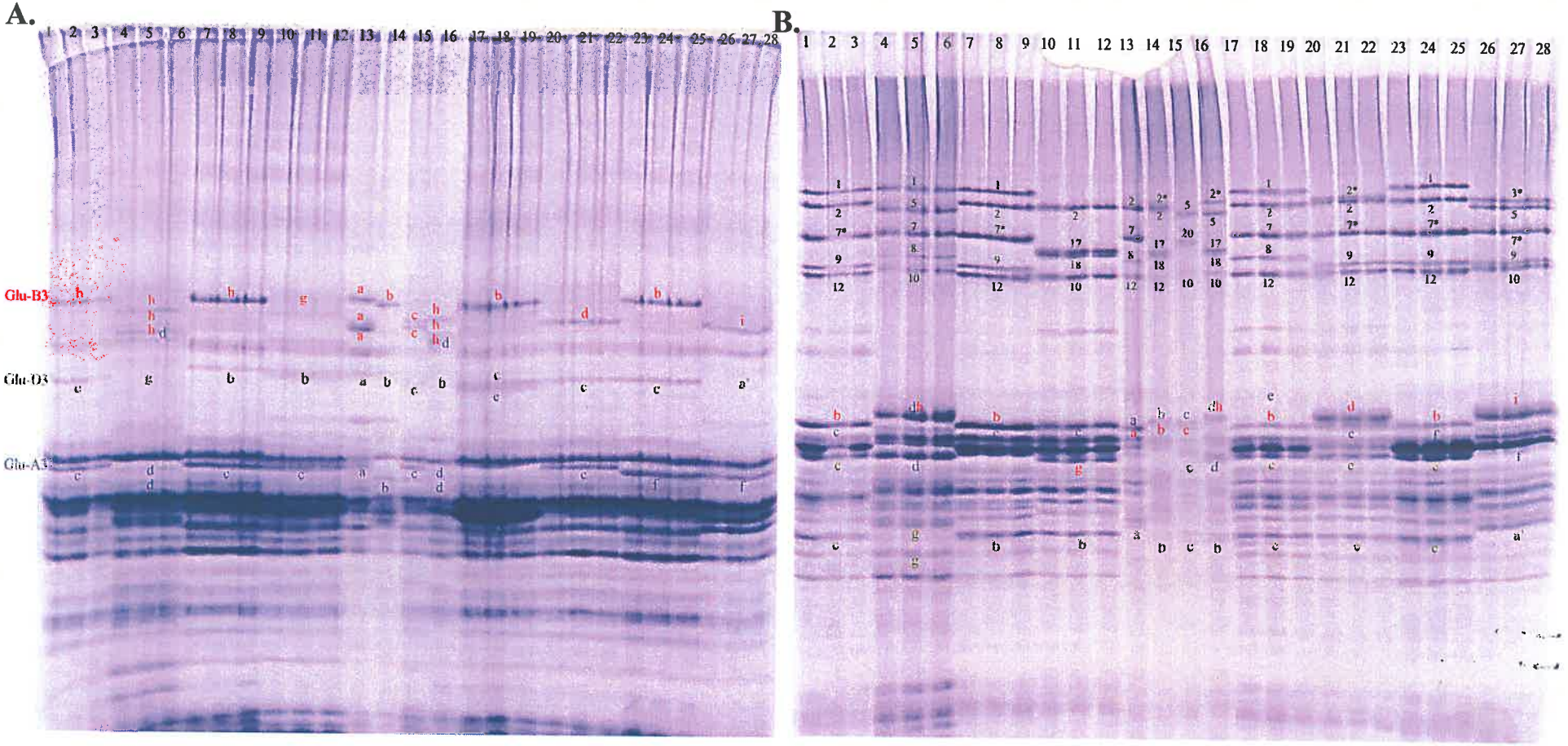

Figure 10. SDS Gradient PAGE (8-12%) of Aroona Isolines **A.** Gliadins and **B.** Glutenins. Lanes 1-3, Aroona; Lanes 4-6, Hira; Lanes 7-9, Aril 31-2; Lanes 10-12, Millewa; Lane 13, Chinese Spring; Lane 14, Gabo; Lane 15, Jabiru; Lane 16, Wilgoyne; Lanes 17-19, DA 7+8 A3e; Lanes 20-22, DA 2* B3d; Lanes 23-25, Aril 20-1; Lanes 26-28, BT 2288.

Year	2000		2001		2002		2003	
Kg/ha of Nitrogen	50		125		100		75	
Flour Protein range (mean)	7.63-8.74%		7.54-11.39%		9.88-13.02%		7.77-9.27%	
Aroona	1.02	**	4.12	***	3.62	***	1.48	***
Aril 10-1, 17+18	0.92	**	3.40	***	2.73	**	1.77	***
Aril 12-3, 3+12	1.35	***	3.60	***	4.20	***	1.50	***
Aril 13-3, 5+10	1.45	***	4.10	***	3.67	***	1.43	***
Aril 14-3, 2.2+12	1.05	**	3.85	***	4.13	***	1.50	***
Aril 15-4, A3a	1.00	**	3.45	***	3.37	***	1.63	***
Aril 16-1, A3b	1.15	**	4.40	***	3.27	***	1.50	***
Aril 18-5, A3d	-		-		2.67	**	1.43	***
Aril 18-9, A3d	1.00	**	3.35	***	3.33	***	1.43	***
Aril 19-2, A3e	0.85	*	3.85	***	2.87	**	1.60	***
Aril 20-1, A3f	1.25	***	4.40	***	3.10	***	1.37	***
Aril 21-2, B3a	0.50		3.70	***	2.43	**	1.57	***
Aril 23-4, B3c	0.36		3.60	***	3.33	***	1.73	***
Aril 2-4, 2*	1.45	***	4.25	***	3.00	***	1.50	***
Aril 24-3, B3d	1.25	***	3.15	***	2.33	**	1.80	***
Aril 26-1, B3f	-		3.65	***	3.83	***	1.40	***
Aril 27-3, B3m	-		-		2.87	**	1.67	***
Aril 27-6, B3g	0.75	*	4.10	***	3.17	***	1.80	***
Aril 28-4, B3h	1.10	**	3.93	***	3.47	***	1.67	***
Aril 29-4, B3i	-		4.00	***	3.63	***	1.43	***
Aril 30-1, D3a	0.43		3.48	***	2.47	**	1.40	***
Aril 3-2, null	0.70		4.55	***	3.10	***	1.73	***
Aril 33-1, D3d	1.50	***	3.80	***	2.53	**	1.37	***
Aril 34-1, D3a-Gli-1	0.85	*	3.50	***	4.00	***	1.53	***
Aril 35-1, D3f	1.20	**	3.95	***	3.07	***	1.40	***
Aril 31-2, D3b	1.05	**	4.05	***	3.83	***	1.40	***
Aril 4-3, 3*	1.60	***	3.90	***	3.53	***	1.33	***
Aril 5-2, 7*	1.50	***	-		2.53	**	1.47	***
Aril 6-4, 7+8	1.65	***	4.55	***	3.60	***	1.50	***
Aril 7-1, 7+8	0.70		3.65	***	3.10	***	1.20	***
Aril 9-3, 6+8*	1.55	***	3.68	***	2.90	***	1.27	***
DA 2*, A3d	0.95	*	3.75	***	2.63	**	1.57	***
DA 2*, A3e	-		-		2.53	**	1.70	***
DA 2*, B3d	-		-		-		1.40	***
DA 2*, B3m	-		-		2.45	**	1.97	***
DA 2.2+12, A3d	1.45	***	3.30	***	3.30	***	1.40	***
DA 3*, D3a-Gli-1	0.95	*	4.00	***	4.37	***	1.20	***
DA 5+10, A3d	-		4.35	***	2.67	**	1.00	**
DA 5+10, A3e	-		3.80	***	4.27	***	1.50	***
DA 5+10, B3c	0.90	*	3.95	***	3.27	***	1.43	***
DA 5+10, B3h	1.35	***	3.60	***	3.17	***	1.40	***
DA 6+8*, B3f	-		-		-		1.50	***
DA 7+8, A3a	0.75	*	3.85	***	3.40	***	1.67	***
DA 7+8, A3d	1.45	***	3.50	***	2.27	**	1.55	***
DA 7+8, A3e	-		-		2.90	***	1.43	***
DA 7+8, B3a	-		-		-		1.57	***
DA 7+8, B3c	1.55	***	3.70	***	4.23	***	1.20	***
DA 7+8, B3d	1.20	**	3.40	***	2.50	**	1.48	***
DA 7+8, B3h	1.15	**	4.25	***	3.33	***	1.47	***
DA 7*, A3d	1.20	**	4.25	***	2.57	**	1.47	***
DA B3d, D3a	-		-		3.20	***	1.37	***
DA null, A3a	-		-		3.45	***	1.43	***
DA null, D3a	1.30	***	4.15	***	3.00	***	1.80	***
TA 6+8*, A3d, B3i	-		-		3.10	***	1.40	***
TA null, 7+8, D3a	-		-		3.08	***	1.63	***

Table 1. Difference in FP (%) between nitrogen treatment groups. * = P≤0.05, ** = P≤0.01, *** = P≤0.001 significance level compared to Aroona; blue = positive effect; red = negative effect.

Year	2000		2001		2002		2003	
Kg/ha of Nitrogen	50		125		100		75	
Flour Protein range (mean)	7.63-8.74%		7.54-11.39%		9.88-13.02%		7.77-9.27%	
Aroona 1	5		36.66	*	24.44		32.22	*
Aril 10-1, 17+18	13.11		40	*	-6.67		3.33	
Aril 12-3, 3+12	20		17.5		20		25	
Aril 13-3, 5+10	5		40	*	0		16.66	
Aril 14-3, 2.2+12	15		20		-3.33		36.67	*
Aril 15-4, A3a	-10		27.5		36.66	*	33.34	*
Aril 16-1, A3b	-5		52.5	**	35	*	26.67	
Aril 18-5, A3d	-		-		23.34		30	
Aril 18-9, A3d	10		32.5		26.67		13.34	
Aril 19-2, A3e	7.5		72.5	***	31.67	*	10	
Aril 20-1, A3f	17.5		50	**	23.33		3.34	
Aril 21-2, B3a	12.48		47.5	**	26.66		16.67	
Aril 23-4, B3c	2.48		47.5	**	40	**	6.66	
Aril 2-4, 2*	-17.5		50	**	11.67		28.33	
Aril 24-3, B3d	15		42.5	*	43.34	**	36.66	*
Aril 26-1, B3f	-		22.5		20		15	
Aril 27-3, B3m	-		-		46.66	**	11.67	
Aril 27-6, B3g	35	**	10		-5		15	
Aril 28-4, B3h	2.5		10		0		-3.33	
Aril 29-4, B3i	-		17.5		40	**	25	
Aril 30-1, D3a	-3.66		43.75	*	28.34	*	-1.67	
Aril 3-2, null	12.5		5		15		35	*
Aril 33-1, D3d	5		52.5	**	25		31.66	*
Aril 34-1, D3a-Gli-1-	20		15		21.66		28.34	
Aril 35-1, D3f	20		75	***	10		36.66	*
Aril 31-2, D3b	25		60	***	40	**	23.33	
Aril 4-3, 3*	2.5		45	*	31.67	*	20	
Aril 5-2, 7*	2.5		-		16.67		-6.67	
Aril 6-4, 7+8	-30	*	5		13.33		10	
Aril 7-1, 7+8	0		2.5		0		13.34	
Aril 9-3, 6+8*	-22.5		16.25		25		-10	
DA 2*, A3d	-17.5		2.5		8.34		-8.34	
DA 2*, A3e	-		-		-		33.33	*
DA 2*, B3d	-		-		16.66		0	
DA 2*, B3m	-		-		30	*	1.67	
DA 2.2+12, A3d	10		12.5		18.33		8.33	
DA 3*, D3a-Gli-1-	2.5		-22.5		3.34		1.67	
DA 5+10, A3d	-		-12.5		-5		25	
DA 5+10, A3e	-		47.5	**	16.67		36.67	*
DA 5+10, B3c	0		75	***	56.66	***	21.67	
DA 5+10, B3h	5		20		-		33.33	*
DA 6+8*, B3f	-		-		6.67		11.67	
DA 7+8, A3d	15		2.5		5		6.67	
DA 7+8, A3e	-		-		0		-3.33	
DA 7+8, B3a	-		-		-		-1.67	
DA 7+8, B3c	7.5		-5		-		-1.67	
DA 7+8, B3d	10		112.5	***	16.67		39.17	*
DA 7+8, B3h	-10		-27.5		-8.34		-15	
DA 7*, A3d	7.5		-7.5		-5		11.67	
DA B3d, D3a	-		-		27.5		16.66	
DA null, A3a	-		-		50	***	-3.33	
DA null, D3a	7.5		25		16.67		5	
TA 6+8*, A3d, B3i	-		-		-8.33		-13.33	
TA null, 7+8, D3a	-		-		21.67		-35	*
TA null, 7+8, D3a	-		-		6.67		8.33	

Table 2. Difference in R_{max} (BU) between nitrogen treatment groups. * = P≤0.05, ** = P≤0.01, *** = P≤0.001 significance level compared to Aroona; blue = positive effect; red = negative effect.

Year	2000		2001		2002		2003	
Kg/ha of Nitrogen	50		125		100		75	
Flour Protein range (mean)	7.63-8.74%		7.54-11.39%		9.88-13.02%		7.77-9.27%	
Aroona 1	2.23	*	6.75	***	5.844	***	2.011	**
Aril 10-1, 17+18	1.071		6.25	***	3.533	*	2.134	**
Aril 12-3, 3+12	3.5	***	5.45	***	6.334	***	2.367	***
Aril 13-3, 5+10	3.45	***	6.2	***	5.867	***	2.267	**
Aril 14-3, 2.2+12	0.55		4.45	***	5.133	***	1.2	
Aril 15-4, A3a	1.05		6.2	***	5.2	***	1.5	*
Aril 16-1, A3b	3.05	**	6.1	***	6.667	***	3.033	***
Aril 18-5, A3d	-		-		4.4	**	2.533	***
Aril 18-9, A3d'	1.1		4.4	***	4.634	***	3.033	***
Aril 19-2, A3e	0.1		5.4	***	4.567	**	2.634	***
Aril 20-1, A3f	2.85	**	5.05	***	2.967	*	1.767	*
Aril 21-2, B3a	1.261		5.2	***	2.466		2.733	***
Aril 23-4, B3c	3.761	***	5.55	***	4.1	**	1.2	
Aril 2-4, 2*	2.7	**	4.25	**	4.3	**	1.833	**
Aril 24-3, B3d	1.9	*	4.6	***	3.766	**	2.767	***
Aril 26-1, B3f	-		4.7	***	4.433	**	1.634	*
Aril 27-3, B3m	-		-		3.1	*	2.667	***
Aril 27-6, B3g	0.65		8.8	***	5.533	***	3	***
Aril 28-4, B3h	1.675		5.175	***	5.033	***	1.866	**
Aril 29-4, B3i	-		6.85	***	1.4		3.667	***
Aril 30-1, D3a	0.642		5.8	***	3.467	*	2.267	**
Aril 3-2, null	1.9	*	6.15	***	4.767	***	3.067	***
Aril 33-1, D3d	2.05	*	5.6	***	4.867	***	2.433	***
Aril 34-1, D3a-Gli-1'	1.65		7.05	***	6.567	***	2.3	**
Aril 35-1, D3f	1.8	*	8.25	***	4.733	***	1.867	**
Aril 36-2, D3b	1.75		7	***	4.8	***	1.334	
Aril 4-3, 3*	2.25	*	7	***	4.066	**	2.6	***
Aril 5-2, 7*	2.4	**	-		3.334	*	2.167	**
Aril 6-4, 7+8	3.05	**	6.4	***	5.634	***	1.866	**
Aril 7-1, 7+8	0.95		4.1	**	1.333		1.833	**
Aril 9-3, 6+8*	2.1	*	4.475	***	2.2		3	***
DA 2*, A3d	0.75		6.9	***	3.6	**	1.634	*
DA 2*, A3e	-		-		3.6	**	1.967	**
DA 2*, B3d	-		-		-		1.95	**
DA 2*, B3m	-		-		3.65	**	3.567	***
DA 2.2+12, A3d	2.9	**	5.55	***	5.533	***	2.633	***
DA 3*, D3a-Gli-1'	1.55		8.15	***	5.9	***	1.633	*
DA 5+10, A3d	-		5.7	***	3.867	**	2.233	**
DA 5+10, A3e	-		5.95	***	6	***	1.567	*
DA 5+10, B3c	0.75		5.85	***	4.233	**	1.334	
DA 5+10, B3h	2.55	**	5.25	***	4.3	**	1.834	**
DA 6+8*, B3f	-		-		-		1.434	*
DA 7+8, A3d	3.25	***	4.7	***	2.7		2.167	**
DA 7+8, A3e	-		-		2.85	*	2.4	***
DA 7+8, B3a	-		-		-		2.266	**
DA 7+8, B3c	1.5		5.7	***	4.5	**	1.833	**
DA 7+8, B3d	-0.45		6.55	***	5.267	***	2.8	***
DA 7+8, B3h	1.45		5.4	***	4.767	***	2.767	***
DA 7*, A3d	2.15	*	6.25	***	4.1	**	2.667	***
DA B3d, D3a	-		-		3.95	**	2.534	***
DA null, A3a	-		-		5.25	***	1.833	**
DA null, D3a	0.7		5.5	***	4.233	**	2.7	***
TA 6+8*, A3d, B3i	-		-		2		3.234	***
TA null, 7*+8, D3a	-		-		3.134	*	2.534	***
TA null, 7+8, D3a	-		-		4.033	**	2.733	***

Table 3. Difference in Ext. (cm) between nitrogen treatment groups. * = P≤0.05, ** = P≤0.01, *** = P≤0.001 significance level compared to Aroona; blue = positive effect; red = negative effect.

Year	2000		2001		2002		2003	
Kg/ha of Nitrogen	50		125		100		75	
Flour Protein range (mean)	7.63-8.74%		7.54-11.39%		9.88-13.02%		7.77-9.27%	
Aroona 1	6.78		32.99	***	38.02	***	15.23	**
Aril 10-1, 17+18	5.53		37.5	***	20.34	*	10.17	*
Aril 12-3, 3+12	15	**	25.38	**	39.41	***	14.83	**
Aril 13-3, 5+10	16.38	***	38	***	37.83	***	18.17	***
Aril 14-3, 2.2+12	7.25		19.63	*	26.84	**	10.34	*
Aril 15-4, A3a	-0.63		32.12	***	37	***	12.17	*
Aril 16-1, A3b	5.87		37.62	***	47.67	***	20.58	***
Aril 18-5, A3d	-		-		34.58	***	17.58	***
Aril 18-9, A3d'	6.37		23	**	33.25	***	17.67	***
Aril 19-2, A3e	2.87		37.62	***	30.34	***	11.25	*
Aril 20-1, A3f	11.62	*	29.75	***	22.33	*	8.17	
Aril 21-2, B3a	5.3		22.13	**	15.08		14.16	**
Aril 23-4, B3c	6.05		23.75	**	21.09	*	3.25	
Aril 2-4, 2*	1.37		24	**	24.42	**	13.91	**
Aril 24-3, B3d	10.37	*	26.75	***	31.58	***	16.84	***
Aril 26-1, B3f	-		19.63	*	22.75	**	9.09	
Aril 27-3, B3m	-		-		27.92	**	13.58	**
Aril 27-6, B3g	12.62	**	35.75	***	32.67	***	17.83	***
Aril 28-4, B3h	3.56		18.13	*	24.84	**	6.5	
Aril 29-4, B3i	-		28.12	***	29.92	***	21.09	***
Aril 30-1, D3a	1.7		32.75	***	25.91	**	9.17	
Aril 3-2, null	6.5		17	*	26	**	16.25	**
Aril 33-1, D3d	7.63		39.5	***	37.67	***	19.83	***
Aril 34-1, D3a-Gli-1	10.63	*	34.37	***	44.42	***	16.83	***
Aril 35-1, D3f	10	*	57.25	***	30.25	***	14.16	**
Aril 36-2, D3b	9.88	*	40.87	***	35.08	***	8.42	
Aril 4-3, 3*	4.63		37.63	***	27.58	**	16.66	***
Aril 5-2, 7*	4.25		-		18.09	*	7.5	
Aril 6-4, 7+8	0.12		23.13	**	34.08	***	10.17	*
Aril 7-1, 7+8	2.88		16.25	*	6.58		9.84	*
Aril 9-3, 6+8*	-1.38		15.38		15.75		8.58	
DA 2*, A3d	-3.63		28.37	***	21.08	*	5.09	
DA 2*, A3e	-		-		20.83	*	11.75	*
DA 2*, B3d	-		-		-		6.88	
DA 2*, B3m	-		-		27.25	**	13.91	**
DA 2.2+12, A3d	10.12	*	24.75	**	35.92	***	14.92	**
DA 3*, D3a-Gli-1	7.13		34.5	***	38.09	***	8.92	
DA 5+10, A3d	-		26.75	***	23.5	**	18.34	***
DA 5+10, A3e	-		43	***	44	***	13.25	**
DA 5+10, B3c	1.88		40.63	***	32.75	***	9.17	
DA 5+10, B3h	9.5	*	23.62	**	26.5	**	15.33	**
DA 6+8*, B3f	-		-		-		6.33	
DA 7+8, A3d	15.63	**	21.12	**	16.08		11.25	*
DA 7+8, A3e	-		-		14		8.33	
DA 7+8, B3a	-		-		-		8.75	
DA 7+8, B3c	5		13.62		20.34	*	5.08	
DA 7+8, B3d	0.13		63.75	***	42	***	21.87	***
DA 7+8, B3h	1.63		8.25		18.25	*	9.17	
DA 7*, A3d	5.37		18.62	*	19	*	13.83	**
DA B3d, D3a	-		-		35.12	***	10.92	*
DA null, A3a	-		-		29.13	**	6.66	
DA null, D3a	2.13		20.62	**	20.92	*	9.33	
TA 6+8*, A3d, B3i	-		-		7.25		8.5	
TA null, 7*+8, D3a	-		-		15.42		1.25	
TA null, 7+8, D3a	-		-		16.75		10.5	*

Table 4. Difference in Area (cm^2) between nitrogen treatment groups. * = P≤0.05, ** = P≤0.01, *** = P≤0.001 significance level compared to Aroona; blue = positive effect; red = negative effect.

Year	2000		2001		2002		2003	
mean Flour Protein	8.19%		9.47%		11.45%		8.52%	
Aroona 1	140.5		210.3		288.4		238.1	
Aril 10-1, 17+18	190.4	***	227.5		296.4		272.3	**
Aril 12-3, 3+12	139.9		208.6		289.1		229.7	
Aril 13-3, 5+10	235.1	***	290.1	***	366.8	***	353.5	***
Aril 14-3, 2.2+12	102.5	***	193.0		267.9	*	214.0	*
Aril 15-4, A3a	119.7	*	221.2		281.5		240.8	
Aril 16-1, A3b	147.8		219.1		302.2		260.7	*
Aril 18-5, A3d	-		-		337.2	***	278.2	***
Aril 18-9, A3d'	147.9		198.6		294.3		256.2	
Aril 19-2, A3e	86.5	***	168.3	**	231.0	***	214.0	*
Aril 20-1, A3f	131.7		194.9		301.7		243.5	
Aril 21-2, B3a	85.4	***	143.8	***	208.3	***	196.3	***
Aril 23-4, B3c	67.8	***	121.4	***	153.0	***	139.3	***
Aril 2-4, 2*	119.0	*	187.7		263.2	*	231.8	
Aril 24-3, B3d	127.5		188.6		262.9	*	209.6	*
Aril 26-1, B3f	-		149.2	***	224.9	***	209.9	*
Aril 27-3, B3m	-		-		279.0		236.5	
Aril 27-6, B3g	117.4	*	227.9		315.4	**	268.1	**
Aril 28-4, B3h	107.4	***	166.2	**	241.8	***	218.5	
Aril 29-4, B3i	-		187.0		218.5	***	217.7	
Aril 30-1, D3a	116.8	*	209.4		279.9		232.3	
Aril 3-2, null	104.0	***	152.7	***	233.8	***	173.4	***
Aril 33-1, D3d	157.7		238.9		331.9	***	285.2	***
Aril 34-1, D3a-Gli-D1'	144.4		252.0	**	326.8	***	291.2	***
Aril 35-1, D3f	160.1	*	254.7	**	316.1	**	259.8	
Aril 36-2, D3b	114.7	**	194.9		252.4	***	209.6	*
Aril 4-3, 3*	139.1		207.7		243.8	***	238.4	
Aril 5-2, 7	86.2	***	-		210.3	***	200.2	***
Aril 6-4, 7+8	130.0		199.9		298.6		267.5	**
Aril 7-1, 7+8	124.8		213.1		263.1	*	223.5	
Aril 9-3, 6+8*	96.5	***	154.3	***	222.9	***	196.3	***
DA 2*, A3d	149.1		230.8		284.3		245.9	
DA 2*, A3e	-		-		232.0	***	186.0	***
DA 2*, B3d	-		-		-		201.1	**
DA 2*, B3m	-		-		262.0	**	221.9	
DA 2.2+12, A3d	129.8		201.1		278.5		251.1	
DA 3*, D3a-Gli-D1'	176.6	***	246.3	*	323.7	***	291.6	***
DA 5+10, A3d	-		300.9	***	388.3	***	357.0	***
DA 5+10, A3e	-		265.8	***	349.5	***	293.7	***
DA 5+10, B3c	124.9		217.8		269.4		265.7	*
DA 5+10, B3h	167.6	**	265.0	***	342.1	***	314.4	***
DA 6+8*, B3f	-		-		-		181.8	***
DA 7*, A3d	86.4	***	173.7	*	245.0	***	226.6	
DA 7+8, A3d	182.6	***	248.7	*	305.0		258.1	
DA 7+8, A3e	-		-		219.3	***	210.7	*
DA 7+8, B3a	-		-		-		192.6	***
DA 7+8, B3c	103.7	***	157.1	***	188.2	***	184.4	***
DA 7+8, B3d	140.2		260.9	***	324.3	***	244.4	
DA 7+8, B3h	113.3	**	169.0	**	232.1	***	224.2	
DA B3d, D3a	-		-		210.0	***	159.1	***
DA null, A3a	-		-		246.8	***	219.0	
DA null, D3a	71.1	***	155.3	***	207.0	***	182.2	***
TA 6+8*, A3d, B3i	-		-		226.9	***	204.9	**
TA null, 7+8, D3a	-		-		176.7	***	171.9	***
TA null, 7+8, D3a	-		-		206.0	***	184.5	***

Table 5. ANOVA maximum resistance (R_{max}, BU) predictions for Aroona isolines (genotype effects) with protein content treated as a co-variate. * = p≤0.05, ** = p≤0.01, *** = p≤0.001 significance level compared to Aroona; blue = positive effect; red = negative effect on R_{max}.

Year mean Flour Protein	2000 8.19%		2001 9.47%		2002 11.45%		2003 8.52%	
Aroona 1	15.63		15.24		15.48		12.21	
Aril 10-1, 17+18	15.46		14.90		16.57		11.16	**
Aril 12-3, 3+12	15.36		14.73		15.79		12.01	
Aril 13-3, 5+10	15.59		14.60		14.93		11.13	**
Aril 14-3, 2.2+12	16.03		15.05		14.83		12.03	
Aril 15-4, A3a	16.11		15.77		15.81		11.69	
Aril 16-1, A3b	15.73		15.77		16.05		11.58	
Aril 18-5, A3d	-		-		15.65		12.08	
Aril 18-9, A3d	16.02		14.56		17.01	*	12.68	
Aril 19-2, A3e	14.43	*	14.24		14.13	*	10.89	***
Aril 20-1, A3f	15.20		13.56	*	15.47		11.28	*
Aril 21-2, B3a	14.75		14.35		15.84		11.80	
Aril 23-4, B3c	14.88		14.85		15.42		11.99	
Aril 2-4, 2*	15.48		14.89		15.14		11.80	
Aril 24-3, B3d	16.50		15.51		16.59		12.43	
Aril 26-1, B3f	-		15.57		16.61		12.30	
Aril 27-3, B3m	-		-		16.53		11.61	
Aril 27-6, B3g	16.09		15.60		16.99	*	11.87	
Aril 28-4, B3h	15.56		15.33		14.62		12.35	
Aril 29-4, B3i	-		16.54	*	18.58	***	13.90	***
Aril 30-1, D3a	15.48		14.89		15.70		12.36	
Aril 3-2, null	13.52	***	13.48	**	13.62	**	11.06	**
Aril 33-1, D3d	15.71		16.18		16.37		12.03	
Aril 34-1, D3a-Gli-D1	15.81		15.78		15.93		11.94	
Aril 35-1, D3f	14.81		15.27		15.67		11.93	
Aril 31-2, D3b	15.62		14.93		15.34		12.05	
Aril 4-3, 3*	14.72		14.89		15.12		11.61	
Aril 5-2, 7*	15.79		-		15.79		12.16	
Aril 6-4, 7+8	15.40		14.49		14.63		11.37	*
Aril 7-1, 7+8	16.12		14.29		15.13		11.36	*
Aril 9-3, 6+8*	15.11		14.25		15.12		11.48	
DA 2*, A3d	15.33		14.70		15.92		11.50	
DA 2*, A3e	-		-		14.93		10.63	***
DA 2*, B3d	-		-		-		12.73	
DA 2*, B3m	-		-		16.36		12.43	
DA 2.2+12, A3d	15.87		15.13		16.20		12.07	
DA 3*, D3a-Gli-D1	16.03		15.34		16.43		11.40	*
DA 5+10, A3d	-		14.11		14.75		11.55	
DA 5+10, A3e	-		13.63	*	13.98	*	9.99	***
DA 5+10, B3c	14.95		14.45		14.81		11.71	
DA 5+10, B3h	15.44		14.29		14.23	*	11.18	**
DA 6+8*, B3f	-		-		-		11.76	
DA 7+8, A3a	15.00		15.93		15.61		11.78	
DA 7+8, A3d	15.47		14.27		15.08		11.53	
DA 7+8, A3e	-		-		14.62		10.62	***
DA 7+8, B3a	-		-		-		11.39	*
DA 7+8, B3c	14.63		14.53		14.32		11.87	
DA 7+8, B3d	16.38		15.59		18.03	***	13.13	*
DA 7+8, B3h	14.81		14.35		15.06		11.59	
DA 7*, A3d	14.83		14.39		15.75		12.35	
DA B3d, D3a	-		-		16.94	*	12.57	
DA null, A3a	-		-		14.05	*	10.94	***
DA null, D3a	15.60		14.32		15.06		11.76	
TA 6+8*, A3d, B3i	-		-		15.75		12.58	
TA null, 7+8, D3a	-		-		14.45		11.55	

Table 6. ANOVA extensibility (Ext., cm) predictions for Aroona isolines (genotype effects) with protein content treated as a co-variate. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ significance level compared to Aroona; blue = positive effect; red = negative effect on Ext.

Year mean Flour Protein	2000 8.19%		2001 9.47%		2002 11.45%		2003 8.52%	
Aroona 1	41.70		56.82		81.54		54.51	
Aril 10-1, 17+18	54.89	***	62.96		91.39	*	55.87	
Aril 12-3, 3+12	40.63		54.25		84.83		50.78	
Aril 13-3, 5+10	66.26	***	73.11	**	96.24	**	70.50	***
Aril 14-3, 2.2+12	31.88	**	53.02		72.91		48.14	*
Aril 15-4, A3a	36.72		61.06		79.29		51.95	
Aril 16-1, A3b	43.39		62.26		89.26		55.77	
Aril 18-5, A3d	-		-		96.11	**	61.59	*
Aril 18-9, A3d	44.30		49.02		92.78	*	60.63	
Aril 19-2, A3e	24.58	***	43.63	*	60.48	***	43.84	***
Aril 20-1, A3f	38.71		48.76		85.03		50.66	
Aril 21-2, B3a	24.44	***	36.93	**	61.27	***	43.82	***
Aril 23-4, B3c	20.38	***	32.52	***	47.23	***	33.59	***
Aril 2-4, 2*	34.58	*	48.80		73.92		51.94	
Aril 24-3, B3d	40.20		50.40		81.32		48.97	
Aril 26-1, B3f	-		43.52	*	68.04	**	48.37	
Aril 27-3, B3m	-		-		84.27		50.45	
Aril 27-6, B3g	35.94		61.52		96.41	**	58.77	
Aril 28-4, B3h	32.13	**	45.83		64.91	***	50.65	
Aril 29-4, B3i	-		55.62		68.50	**	57.96	
Aril 30-1, D3a	34.22	*	55.77		82.48		54.03	
Aril 3-2, null	26.98	***	37.13	**	59.29	***	37.93	***
Aril 33-1, D3d	45.79		70.82	*	99.72	***	63.15	**
Aril 34-1, D3a-Gli-D1$^-$	42.06		70.01	*	95.04	**	64.29	**
Aril 35-1, D3f	44.17		71.92	*	89.51		56.73	
Aril 31-2, D3b	34.25	*	53.61		70.64	*	47.21	*
Aril 4-3, 3*	38.73		55.48		69.13	*	53.34	
Aril 5-2, 7*	27.48	***	-		61.25	***	46.04	**
Aril 6-4, 7+8	37.39		52.04		77.76		55.51	
Aril 7-1, 7+8	38.04		53.51		73.63		47.62	*
Aril 9-3, 6+8*	27.39	***	38.99	**	61.59	***	41.40	***
DA 2*, A3d	43.39		59.43		81.40		52.87	
DA 2*, A3e	-		-		63.52	***	38.44	***
DA 2*, B3d	-		-		-		48.01	*
DA 2*, B3m	-		-		79.69		52.36	
DA 2.2+12, A3d	38.82		54.43		84.33		56.39	
DA 3*, D3a-Gli-D1$^-$	52.64	***	68.41		96.11	**	61.37	*
DA 5+10, A3d	-		74.97	**	103.67	***	77.33	***
DA 5+10, A3e	-		66.08		88.36		53.82	
DA 5+10, B3c	35.35		56.22		71.53	*	57.21	
DA 5+10, B3h	48.32	*	64.73		85.40		64.58	**
DA 6+8*, B3f	-		-		-		40.70	***
DA 7+8, A3a	27.32	***	54.79		68.58	**	48.72	
DA 7+8, A3d	51.83	**	61.15		80.75		55.50	
DA 7+8, A3e	-		-		59.17	***	42.34	***
DA 7+8, B3a	-		-		-		40.79	***
DA 7+8, B3c	29.20	***	39.54	**	50.93	***	41.11	***
DA 7+8, B3d	42.86		74.05	**	109.09	***	60.59	
DA 7+8, B3h	32.40	**	43.13	*	63.90	***	48.98	
DA 7*, A3d	24.73	***	43.31	*	70.95	*	52.24	
DA B3d, D3a	-		-		67.58	**	39.38	***
DA null, A3a	-		-		64.82	***	45.03	**
DA null, D3a	22.66	***	41.10	**	60.35	***	40.56	***
TA 6+8*, A3d, B3i	-		-		65.37	**	48.70	
TA null, 7+8, D3a	-		-		52.19	***	39.53	***

Table 7. ANOVA dough strength (Area, cm^2) predictions for Aroona isolines (genotype effects) with protein content treated as a co-variate. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ significance level compared to Aroona; blue = positive effect; red = negative effect on Area.

Glutenin Allele	Frequency (n =)	Glutenin Allele	Frequency (n =)
Glu-A1		***Glu-A3***	
a	866	*a*	48
b	66	*b*	20
c	74	*c*	753
p	40	*d*	146
		e	59
		f	20
Glu-B1		***Glu-B3***	
a	36	*a*	25
b	190	*b*	741
c	758	*c*	60
d	42	*d*	60
i	20	*f*	22
		g	20
		h	68
		i	28
		m	22
Glu-D1		***Glu-D3***	
a	894	*a*	90
b	20	*a-GliD1- (a')*	40
d	92	*b*	20
f	40	*c*	864
		d	12
		f	20

Table 1. Glutenin allele frequencies included in the REML analysis which are summarised in Tables 2, 3 and 4.

		Glu-A1				*Glu-B1*					*Glu-D1*				*Glu-A3*						*Glu-B3*								
		a	*b*	*c*	*p*	*a*	*b*	*c*	*d*	*i*	*a*	*b*	*d*	*f*	*a*	*b*	*c*	*d*	*e*	*f*	*a*	*b*	*c*	*d*	*f*	*g*	*h*	*i*	*m*
Glu-B1		194	178	146	187	134	192	190	151	214	160	158	251	135	173	193	178	200	134	178	153	205	120	180	167	222	169	170	199
a	134	151	136	103	144	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
b	192	210	194	162	202	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
c	190	208	192	159	200	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
d	151	169	153	121	162	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
i	214	231	216	183	224	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		Ave. SED = 21.4																											
Glu-D1																													
a	160	178	162	130	170	118	176	174	135	197	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
b	158	176	160	128	169	116	174	172	133	196	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
d	251	269	253	220	261	208	267	265	226	288	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
f	135	153	137	105	146	93	151	149	110	173	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		Ave. SED = 21.3				Ave. SED = 23.0																							
Glu-A3																													
a	173	191	175	143	184	131	189	187	148	211	157	155	248	132	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
b	193	211	195	163	204	151	209	207	168	231	177	175	268	152	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
c	178	196	180	148	189	136	194	192	153	216	162	160	253	138	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
d	200	218	202	170	211	158	216	214	175	238	184	182	275	160	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
e	134	152	136	104	145	92	150	148	109	172	118	116	209	93	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
f	178	196	180	147	188	135	194	192	153	215	162	160	252	137	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		Ave. SED = 21.9				Ave. SED = 23.6					Ave. SED = 23.5																		
Glu-B3																													
a	153	171	155	123	163	111	169	167	128	190	137	135	228	112	150	170	155	177	111	155	*	*	*	*	*	*	*	*	*
b	205	223	207	174	215	162	221	219	180	242	189	187	280	164	202	222	207	229	163	207	*	*	*	*	*	*	*	*	*
c	120	137	122	89	130	77	135	133	95	157	103	102	194	79	117	137	122	144	78	121	*	*	*	*	*	*	*	*	*
d	180	198	182	150	191	138	196	194	155	218	164	162	255	139	177	197	182	204	138	182	*	*	*	*	*	*	*	*	*
f	167	185	169	136	177	124	183	181	142	204	151	149	242	126	164	184	169	191	125	168	*	*	*	*	*	*	*	*	*
g	222	240	224	192	233	180	238	236	197	260	206	204	297	182	219	239	224	246	180	224	*	*	*	*	*	*	*	*	*
h	169	187	171	139	180	127	185	183	145	207	153	151	244	129	166	187	172	194	127	171	*	*	*	*	*	*	*	*	*
i	170	188	172	139	180	127	186	184	145	207	154	152	245	129	167	187	172	194	128	171	*	*	*	*	*	*	*	*	*
m	199	216	201	168	209	156′	215	212	174	236	183	181	273	158	196	216	201	223	157	200	*	*	*	*	*	*	*	*	*
		Ave. SED = 22.6				Ave. SED = 24.1					Ave. SED = 24.1				Ave. SED = 24.7														
Glu-D3																													
a	158	176	160	128	169	116	174	172	133	196	142	140	233	118	155	175	160	182	116	160	135	187	102	162	149	204	152	152	181
a'	217	234	219	186	227	174	232	230	192	254	200	199	291	176	213	234	219	241	174	218	193	245	160	221	207	263	210	210	239
b	147	164	149	116	157	104	162	160	122	184	130	129	221	106	144	164	149	171	105	148	123	175	90	151	137	193	140	140	169
c	174	191	176	143	184	131	189	187	149	211	157	156	248	133	170	191	176	198	131	175	150	202	117	178	164	220	167	167	196
d	160	178	162	130	171	118	176	174	135	198	144	142	235	120	157	177	162	185	118	162	137	189	104	164	151	207	154	154	183
f	201	219	203	170	211	159	217	215	176	238	185	183	276	160	198	218	203	225	159	203	178	230	144	205	192	247	194	195	223
		Ave. SED = 23.0				Ave. SED = 24.6					Ave. SED = 24.6				Ave. SED = 25.1						Ave. SED = 25.7								

Table 2. Two-way REML predictions for maximum resistance ($\mathbf{R_{max}}$). Treatment groups combined; mean FP is 9.4%. Actual FP treated as a covariate; allele main effects in red; shaded highlights the allele combination present in Aroona.

		Glu-A1				*Glu-B1*					*Glu-D1*				*Glu-A3*						*Glu-B3*								
		a	***b***	***c***	***p***	***a***	***b***	***c***	***d***	***i***	***a***	***b***	***d***	***f***	***a***	***b***	***c***	***d***	***e***	***f***	***a***	***b***	***c***	***d***	***f***	***g***	***h***	***i***	***m***
Glu-B1		**14.8**	**14.7**	**13.7**	**14.5**	**14.7**	**14.4**	**14.6**	**13.8**	**14.7**	**14.6**	**14.6**	**14.0**	**14.6**	**14.8**	**14.7**	**14.6**	**14.7**	**13.6**	**14.0**	**13.9**	**14.1**	**14.0**	**15.2**	**14.8**	**14.8**	**14.0**	**15.5**	**14.7**
a	14.7	15.1	15.0	14.0	14.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
b	14.4	14.8	14.7	13.7	14.5	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
c	14.6	15.0	14.9	13.9	14.7	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
d	13.8	14.1	14.0	13.0	13.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
i	14.7	15.0	14.9	13.9	14.7	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		Ave. SED = 0.38																											
Glu-D1																													
a	14.6	14.9	14.8	13.8	14.6	14.8	14.6	14.8	13.9	14.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
b	14.6	14.9	14.9	13.8	14.7	14.8	14.6	14.8	13.9	14.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
d	14.0	14.4	14.3	13.3	13.9	14.3	14.0	14.2	13.3	14.2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
f	14.6	15.0	14.9	13.9	14.7	14.9	14.6	14.8	13.9	14.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		Ave. SED = 0.37				Ave. SED = 0.40																							
Glu-A3																													
a	14.8	15.2	15.1	14.1	14.9	15.1	14.8	15.0	14.1	15.0	14.9	14.9	14.4	15.0	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
b	14.7	15.2	15.1	14.1	14.9	15.1	14.9	15.1	14.2	15.1	15.0	15.0	14.4	15.0	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
c	14.6	15.0	14.9	13.9	14.7	14.9	14.6	14.8	14.0	14.8	14.8	14.8	14.2	14.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
d	14.7	15.0	14.9	13.9	14.7	14.9	14.7	14.9	14.0	14.9	14.8	14.8	14.2	14.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
e	13.6	14.0	13.9	12.9	13.7	13.9	13.6	13.8	13.0	13.8	13.8	13.8	13.2	13.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
f	14.0	14.4	14.3	13.3	14.1	14.3	14.0	14.2	13.3	14.2	14.1	14.2	13.6	14.2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		Ave. SED = 0.39				Ave. SED = 0.41					Ave. SED = 0.41																		
Glu-B3																													
a	13.9	14.2	14.2	13.2	14.0	14.1	13.9	14.0	13.3	14.1	14.0	14.0	13.4	14.1	14.3	14.3	14.0	14.1	13.1	13.5	*	*	*	*	*	*	*	*	*
b	14.1	14.5	14.4	13.4	14.2	14.4	14.1	14.3	13.4	14.2	14.2	14.3	13.7	14.3	14.5	14.6	14.3	14.4	13.3	13.7	*	*	*	*	*	*	*	*	*
c	14.0	14.3	14.3	13.2	14.1	14.2	13.9	14.1	13.4	14.2	14.1	14.1	13.5	14.1	14.3	14.4	14.1	14.2	13.2	13.6	*	*	*	*	*	*	*	*	*
d	15.2	15.5	15.4	14.4	15.3	15.4	15.1	15.3	14.6	15.4	15.3	15.3	14.7	15.3	15.5	15.6	15.3	15.4	14.4	14.8	*	*	*	*	*	*	*	*	*
f	14.8	15.1	15.1	14.2	14.9	15.0	14.8	15.0	14.2	15.0	14.9	14.9	14.3	15.0	15.2	15.2	14.9	15.0	14.0	14.4	*	*	*	*	*	*	*	*	*
g	14.8	15.1	15.1	14.2	14.9	15.0	14.8	15.0	14.2	15.0	14.9	15.0	14.3	15.0	15.2	15.2	15.0	15.0	14.0	14.4	*	*	*	*	*	*	*	*	*
h	14.0	14.4	14.3	13.3	14.1	14.2	14.0	14.2	13.4	14.2	14.1	14.2	13.6	14.2	14.4	14.4	14.2	14.2	13.2	13.6	*	*	*	*	*	*	*	*	*
i	15.5	15.8	15.8	14.8	15.6	15.7	15.5	15.7	14.9	15.7	15.6	15.7	15.1	15.7	15.9	15.9	15.7	15.7	14.7	15.1	*	*	*	*	*	*	*	*	*
m	14.7	15.0	14.9	13.9	14.7	14.9	14.6	14.8	14.1	14.9	14.8	14.8	14.2	14.8	15.0	15.1	14.8	14.9	13.9	14.2	*	*	*	*	*	*	*	*	*
		Ave. SED = 0.38				Ave. SED = 0.40					Ave. SED = 0.40				Ave. SED = 0.41														
Glu-D3																													
a	14.6	14.9	14.9	13.8	14.6	14.8	14.6	14.8	13.9	14.8	14.7	14.7	14.1	14.7	14.9	15.0	14.8	14.8	13.8	14.1	14.0	14.3	14.1	15.3	14.9	14.9	14.1	15.6	14.8
a'	14.7	15.0	15.0	13.9	14.7	14.9	14.7	14.9	14.0	14.9	14.8	14.8	14.2	14.9	15.0	15.1	14.9	14.9	13.9	14.3	14.1	14.4	14.2	15.4	15.0	15.0	14.2	15.7	14.9
b	14.2	14.5	14.5	13.4	14.2	14.4	14.2	14.4	13.5	14.4	14.3	14.3	13.7	14.4	14.5	14.6	14.4	14.4	13.4	13.8	13.6	13.9	13.7	14.9	14.5	14.5	13.7	15.2	14.4
c	14.2	14.6	14.5	13.5	14.3	14.5	14.2	14.4	13.5	14.4	14.3	14.4	13.8	14.4	14.6	14.6	14.4	14.4	13.4	13.8	13.6	13.9	13.7	14.9	14.5	14.5	13.8	15.3	14.4
d	14.8	15.2	15.1	14.1	14.9	15.1	14.8	15.1	14.2	15.1	15.0	15.0	14.4	15.0	15.2	15.3	15.0	15.1	14.0	14.4	14.3	14.5	14.4	15.6	15.2	15.2	14.4	15.9	15.1
f	14.2	14.5	14.4	13.4	14.2	14.4	14.2	14.4	13.5	14.4	14.3	14.3	13.7	14.3	14.5	14.6	14.4	14.4	13.4	13.7	13.6	13.9	13.7	14.9	14.5	14.5	13.7	15.2	14.4
		Ave. SED = 0.43				Ave. SED = 0.45					Ave. SED = 0.45				Ave. SED = 0.46						Ave. SED = 0.45								

Table 3. Two-way REML predictions for extensibility (**Ext.**). Treatment groups combined; mean FP is 9.4%. Actual FP treated as a covariate; allele main effects in red; shaded highlights the allele combination present in Aroona.

		Glu-A1				*Glu-B1*					*Glu-D1*				*Glu-A3*						*Glu-B3*								
		a	*b*	*c*	*p*	*a*	*b*	*c*	*d*	*i*	*a*	*b*	*d*	*f*	*a*	*b*	*c*	*d*	*e*	*f*	*a*	*b*	*c*	*d*	*f*	*g*	*h*	*i*	*m*
Glu-B1		54.2	49.8	38.8	51.8	38.7	52.0	52.7	39.8	60.0	45.3	45.9	63.2	40.2	48.7	54.8	49.6	55.4	35.7	47.7	39.4	54.3	33.9	55.0	48.3	61.0	44.4	51.3	55.6
a	38.7	44.2	39.8	28.9	41.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
b	52.0	57.6	53.2	42.2	55.2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
c	52.7	58.3	53.9	42.9	55.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
d	39.8	45.4	41.0	30.0	50.2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
i	60.0	65.6	61.2	50.2	63.2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		Ave. SED = 5.66																											
Glu-D1																													
a	45.3	50.9	46.5	35.5	48.5	35.4	48.7	49.4	36.5	56.7	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
b	45.9	51.4	47.1	36.1	49.0	35.9	49.3	50.0	37.1	57.3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
d	63.2	68.8	64.4	53.4	66.4	53.3	66.6	67.3	54.4	74.6	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
f	40.2	45.7	41.3	30.3	43.3	30.2	43.5	44.2	31.3	51.5	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		Ave. SED = 5.64				Ave. SED = 6.08																							
Glu-A3																													
a	48.7	54.2	49.8	38.9	51.8	38.7	52.1	52.7	39.9	60.1	45.4	45.9	63.3	40.2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
b	54.8	60.3	56.0	45.0	57.9	44.8	58.2	58.9	46.0	66.2	51.5	52.0	69.4	46.3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
c	49.6	55.2	50.8	39.8	52.8	39.7	53.0	53.7	40.8	61.0	46.3	46.9	64.2	41.1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
d	55.4	60.9	56.5	45.6	58.5	45.4	58.8	59.4	46.6	66.8	52.1	52.6	70.0	46.9	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
e	35.7	41.3	36.9	25.9	38.9	25.8	39.1	39.8	26.9	47.1	32.4	33.0	50.3	27.2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
f	47.7	53.2	48.8	37.9	50.8	37.7	51.1	51.7	38.8	59.1	44.4	44.9	62.3	39.2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		Ave. SED = 5.82				Ave. SED = 6.25					Ave. SED = 6.23																		
Glu-B3																													
a	39.1	44.7	40.3	29.3	42.2	29.1	42.5	43.2	30.3	50.5	35.8	36.4	53.7	30.6	39.1	45.3	40.1	45.8	26.2	38.1	*	*	*	*	*	*	*	*	*
b	53.7	59.3	54.9	43.9	56.9	43.8	57.1	57.8	44.9	65.1	50.4	51.0	68.3	45.2	53.8	59.9	54.7	60.5	40.8	52.8	*	*	*	*	*	*	*	*	*
c	33.2	38.7	34.3	23.3	36.3	23.2	36.5	37.2	24.3	44.5	29.8	30.4	47.7	24.7	33.2	39.3	34.1	39.9	20.2	32.2	*	*	*	*	*	*	*	*	*
d	53.3	58.8	54.4	43.4	56.4	43.3	56.6	57.3	44.4	64.7	49.9	50.5	67.8	44.8	53.3	59.4	54.2	60.0	40.3	52.3	*	*	*	*	*	*	*	*	*
f	47.9	53.5	49.1	38.1	51.1	38.0	51.3	52.0	39.1	59.3	44.6	45.2	62.5	39.4	48.0	54.1	48.9	54.7	35.0	47.0	*	*	*	*	*	*	*	*	*
g	60.7	66.2	61.8	50.8	63.8	50.7	64.1	64.7	51.8	72.1	57.4	57.9	75.2	52.2	60.7	66.8	61.6	67.4	47.8	59.7	*	*	*	*	*	*	*	*	*
h	44.1	49.7	45.3	34.3	47.2	34.1	47.5	48.2	35.3	55.5	40.8	41.4	58.7	35.6	44.2	50.3	45.1	50.8	31.2	43.1	*	*	*	*	*	*	*	*	*
i	50.7	56.3	51.9	40.9	53.8	40.7	54.1	54.8	41.9	62.1	47.4	48.0	65.3	42.2	50.8	56.9	51.7	57.5	37.8	49.7	*	*	*	*	*	*	*	*	*
m	55.2	60.7	56.3	45.3	58.3	45.2	58.6	59.2	46.3	66.6	51.9	52.4	69.7	46.7	55.2	61.3	56.2	61.9	42.3	54.2	*	*	*	*	*	*	*	*	*
		Ave. SED = 6.04				Ave. SED = 6.45					Ave. SED = 6.42				Ave. SED = 6.61														
Glu-D3																													
a	44.5	50.0	45.7	34.7	47.6	34.5	47.9	48.6	35.7	55.9	41.2	41.7	59.1	36.0	44.5	50.6	45.5	51.2	31.6	43.5	35.0	49.6	29.0	49.1	43.8	56.5	40.0	46.6	51.0
a'	59.1	64.6	60.2	49.2	62.2	49.1	62.4	63.1	50.2	70.4	55.7	56.3	73.6	50.6	59.1	65.2	60.0	65.8	46.1	58.1	49.5	64.1	43.6	63.7	58.3	71.1	54.5	61.1	65.6
b	40.2	45.7	41.3	30.3	43.3	30.2	43.6	44.2	31.3	51.6	36.9	37.4	54.7	31.7	40.2	46.3	41.2	46.9	27.3	39.2	30.6	45.2	24.7	44.8	39.5	52.2	35.6	42.2	46.7
c	46.4	51.9	47.5	36.5	49.5	36.4	49.7	50.4	37.5	57.7	43.0	43.6	60.9	37.9	46.4	52.5	47.3	53.1	33.4	45.4	36.8	51.4	30.9	51.0	45.7	58.4	41.8	48.4	52.9
d	48.0	53.5	49.1	38.2	51.1	38.0	51.4	52.0	39.2	59.4	44.7	45.2	62.6	39.5	48.0	54.1	49.0	54.7	35.1	47.0	38.4	53.1	32.5	52.6	47.3	60.0	43.4	50.1	54.5
f	53.8	59.4	55.0	44.0	57.0	43.9	57.2	57.9	45.0	65.2	50.5	51.1	68.4	45.3	53.9	60.0	54.8	60.6	40.9	52.9	44.3	58.9	38.3	58.4	53.1	65.8	49.3	55.9	60.4
		Ave. SED = 6.12				Ave. SED = 6.61					Ave. SED = 6.59				Ave. SED = 6.75						Ave. SED = 6.93								

Table 4. Two-way REML predictions for dough strength (**Area**). N treatment groups combined; mean FP is 9.4%. Actual FP treated as a covariate; allele main effects in red; shaded highlights the allele combination present in Aroona.

Vawser, M-J. & Cornish, G. B. (2004). Over-expression of HMW glutenin subunit Glu-B1 7x in hexaploid wheat varieties (Triticum aestivum). *Australian Journal of Agricultural Research, 55*(5), 577-588.

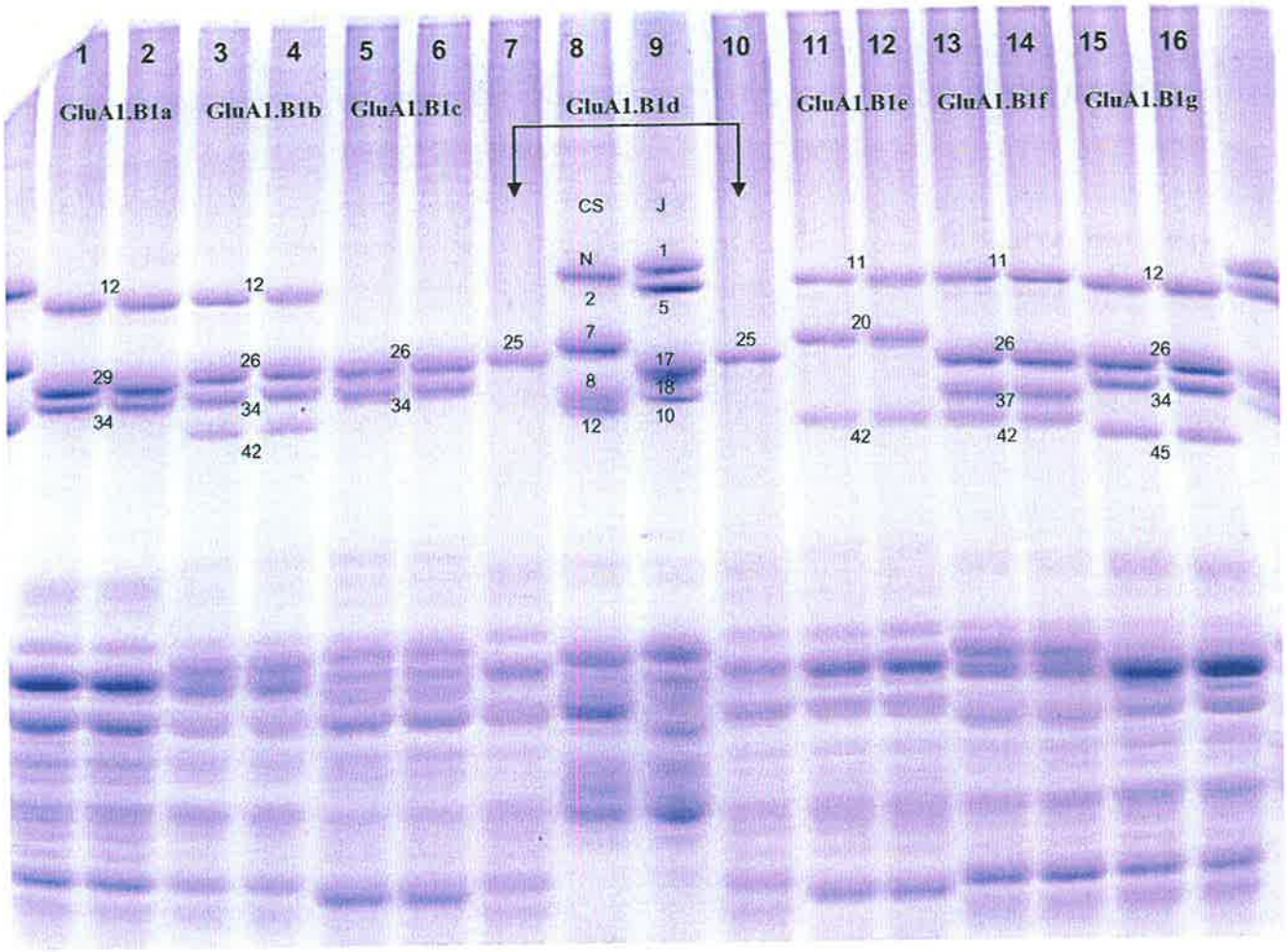

Figure 1. SDS-PAGE (8.5%) of *T.dicoccoides* accessions summarising HMW-GS banding patterns (GluA1.B1 a to g). Lanes 1 & 2: AUS 21322; Lanes 3 & 4: AUS 21318; Lanes 5 & 6: AUS 21312; Lanes 7 & 10: AUS 21288; Lane 8: Chinese Spring; Lane 9: Jabiru; Lanes 11 & 12: AUS 21311; Lanes 13 & 14: AUS 21306; and Lanes 15 & 16: AUS 21317.

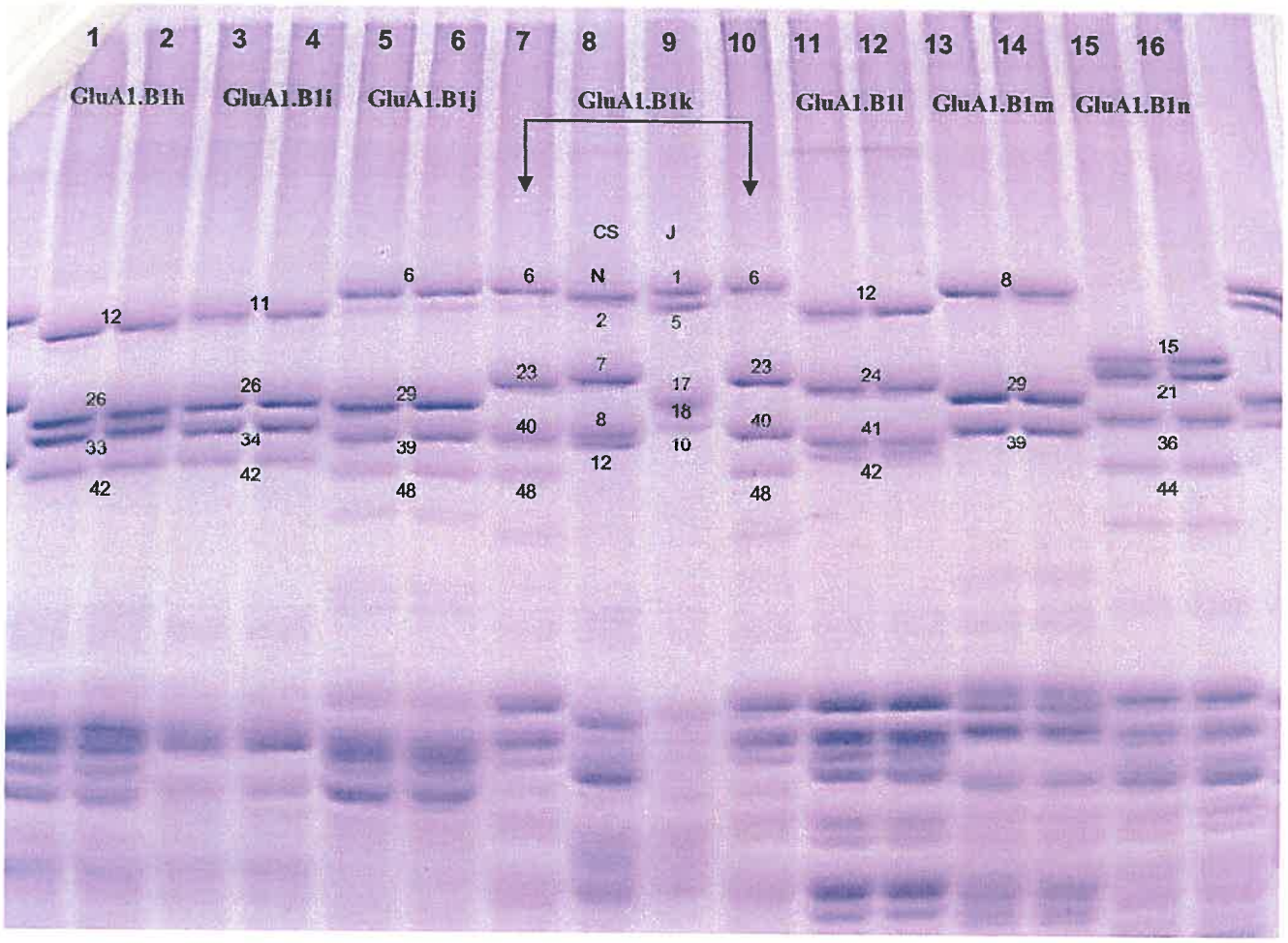

Figure 2. SDS-PAGE (8.5%) of *T.dicoccoides* accessions summarising HMW-GS banding patterns (GluA1.B1 h to n). Lanes 1 & 2: AUS 21420; Lanes 3 & 4: AUS 21426; Lanes 5 & 6: AUS 21432; Lanes 7 & 10: AUS 21433; Lane 8: Chinese Spring; Lane 9: Jabiru; Lanes 11 & 12: AUS 21436; Lanes 13 & 14: AUS 21447; and Lanes 15 & 16: AUS 21443.

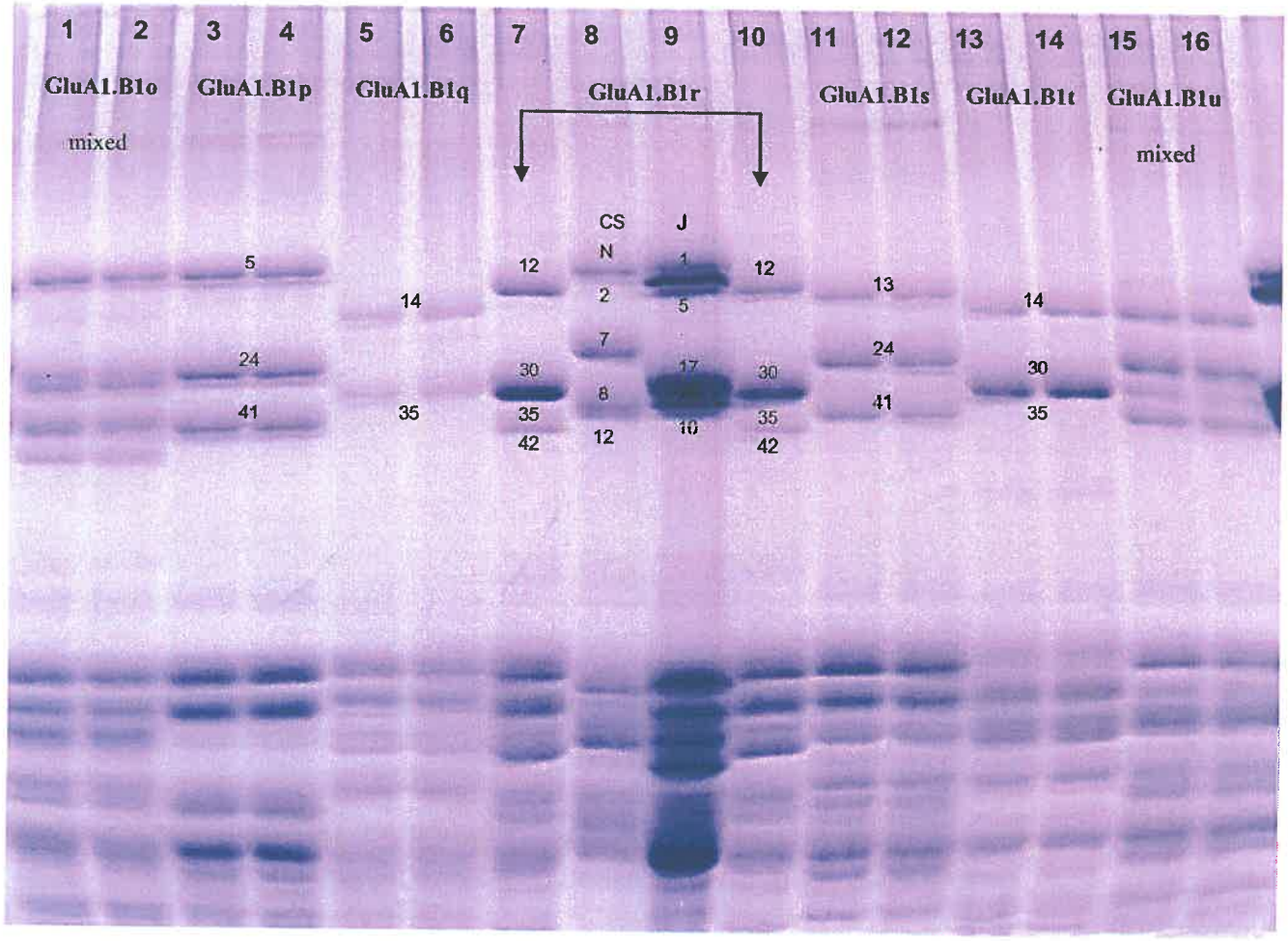

Figure 3. SDS-PAGE (8.5%) of *T.dicoccoides* accessions summarising HMW-GS banding patterns (GluA1.B1 o to u). Lanes 1 & 2: AUS 21449; Lanes 3 & 4: AUS 21453; Lanes 5 & 6: AUS 21463; Lanes 7 & 10: AUS 21478; Lane 8: Chinese Spring; Lane 9: Jabiru; Lanes 11 & 12: AUS 21479; Lanes 13 & 14: AUS 21481; and Lanes 15 & 16: AUS 21483.

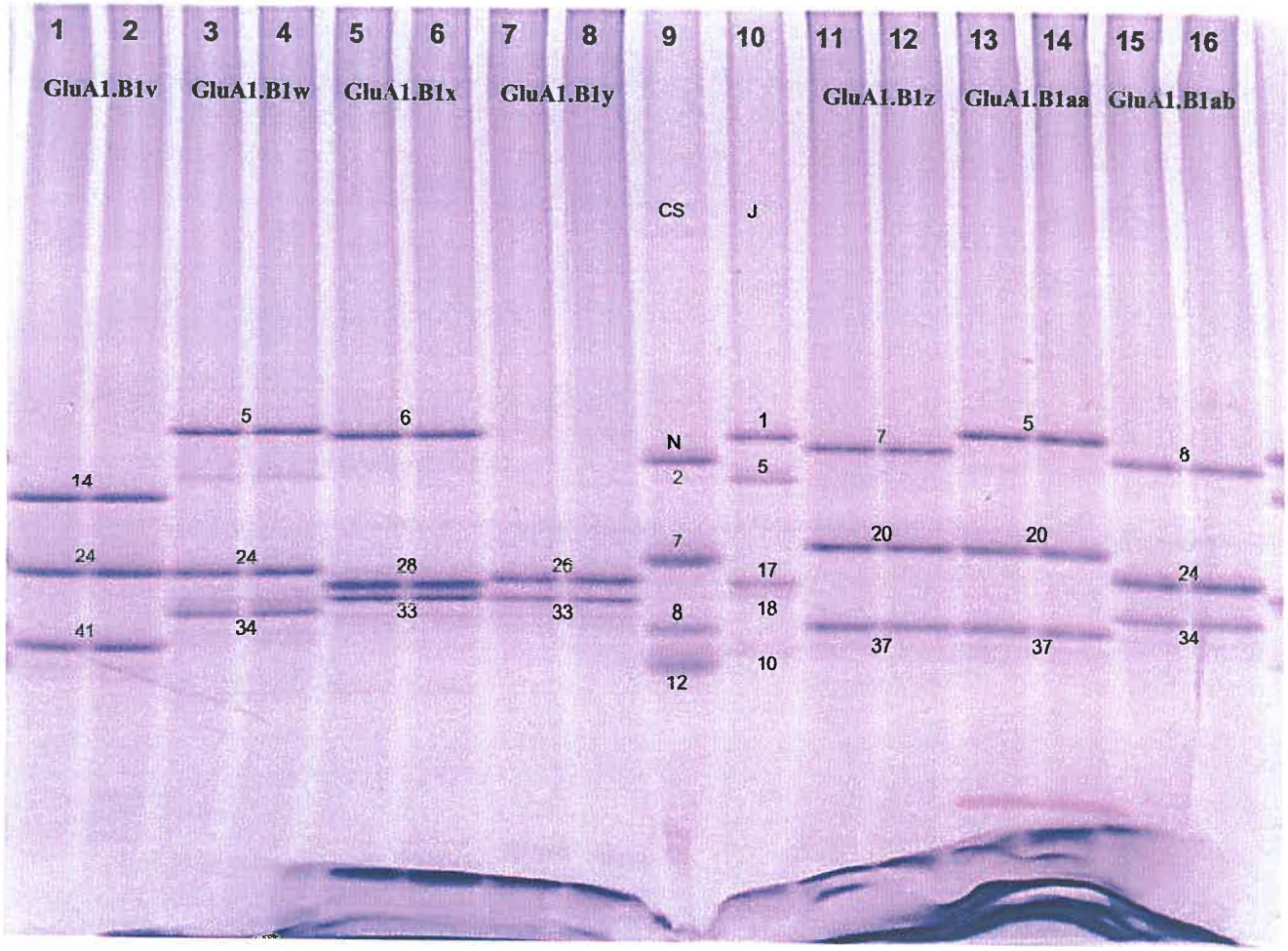

Figure 4. SDS-PAGE (8.5%) of *T.dicoccoides* accessions summarising HMW-GS banding patterns (GluA1.B1 v to ab). Lanes 1 & 2: AUS 21484; Lanes 3 & 4: AUS 21497; Lanes 5 & 6: AUS 21501; Lanes 7 & 8: AUS 21504; Lane 9: Chinese Spring; Lane 10: Jabiru; Lanes 11 & 12: AUS 21515; Lanes 13 & 14: AUS 21516; and Lanes 15 & 16: AUS 22282.

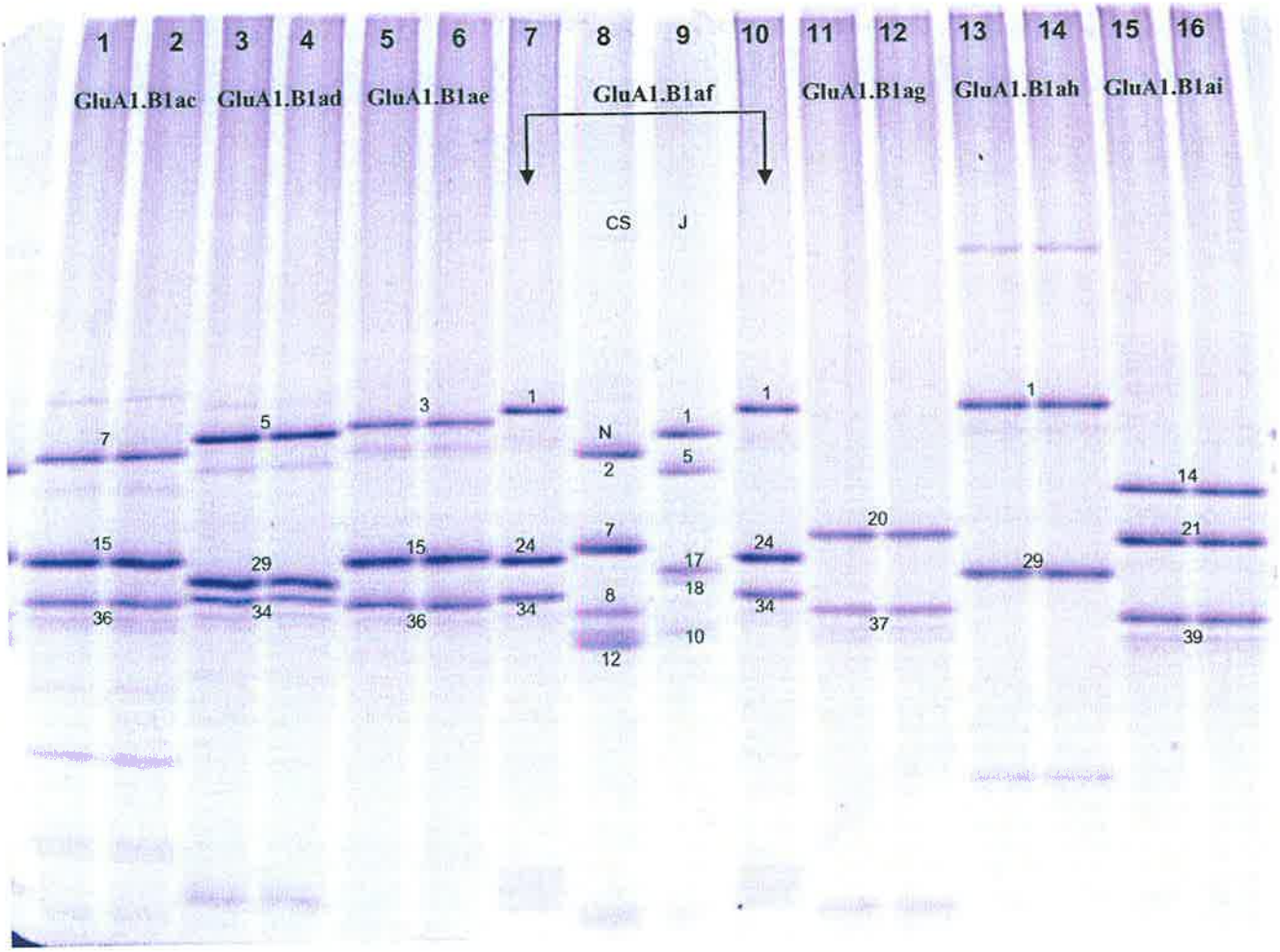

Figure 5. SDS-PAGE (8.5%) of *T.dicoccoides* accessions summarising HMW-GS banding patterns (GluA1.B1 ac to ai). Lanes 1 & 2: AUS 22281; Lanes 3 & 4: AUS 23140; Lanes 5 & 6: AUS 22283; Lanes 7 & 10: AUS 21519; Lane 8: Chinese Spring; Lane 9: Jabiru; Lanes 11 & 12: AUS 23142; Lanes 13 & 14: AUS 23145; and Lanes 15 & 16: AUS 23149.

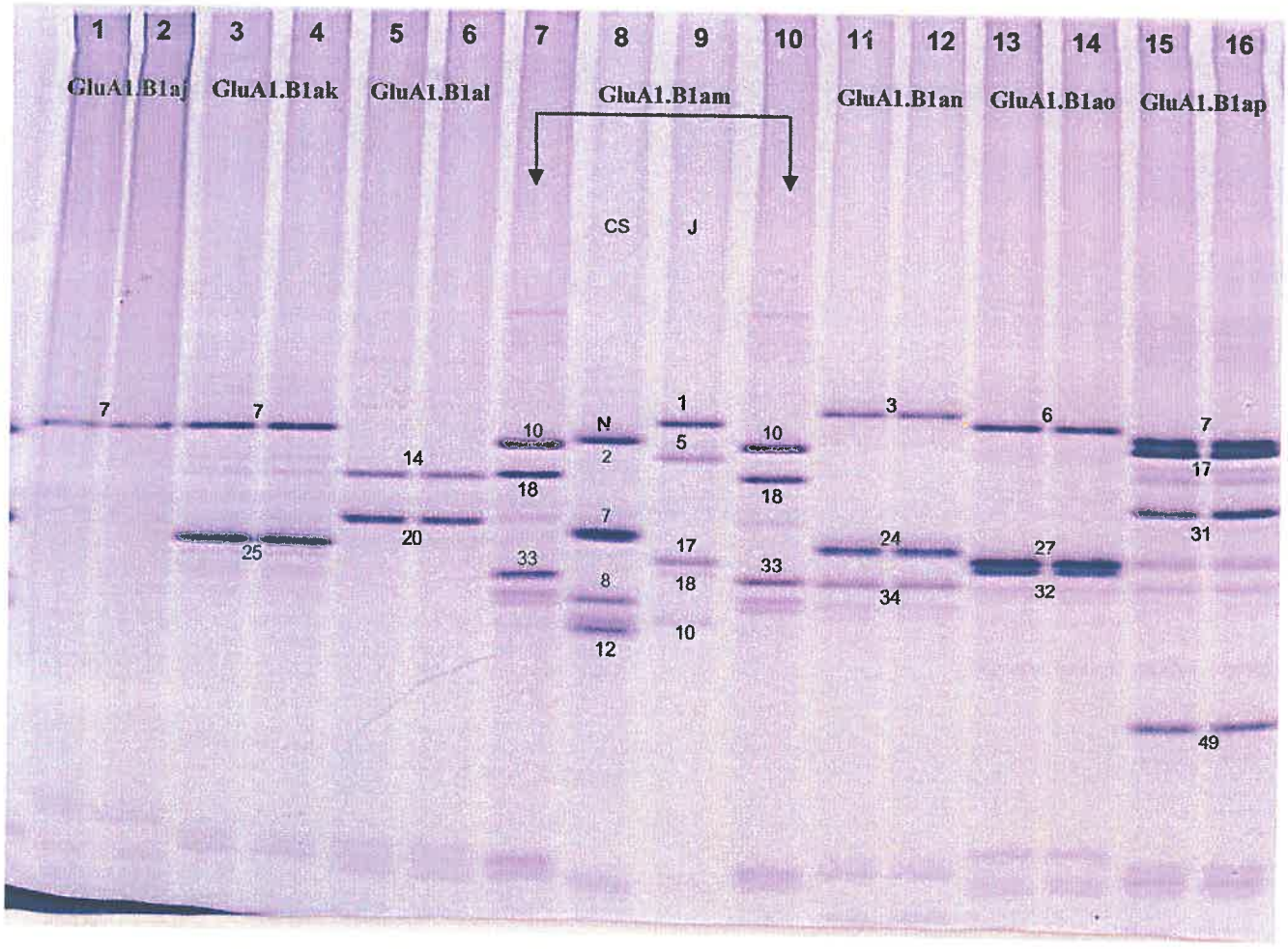

Figure 6. SDS-PAGE (8.5%) of *T.dicoccoides* accessions summarising HMW-GS banding patterns (GluA1.B1 aj to ap). Lanes 1 & 2: AUS 23153; Lanes 3 & 4: AUS 23144; Lanes 5 & 6: AUS 27008; Lanes 7 & 10: AUS 27013; Lane 8: Chinese Spring; Lane 9: Jabiru; Lanes 11 & 12: AUS 22285; Lanes 13 & 14: AUS 21502; and Lanes 15 & 16: AUS 27017.

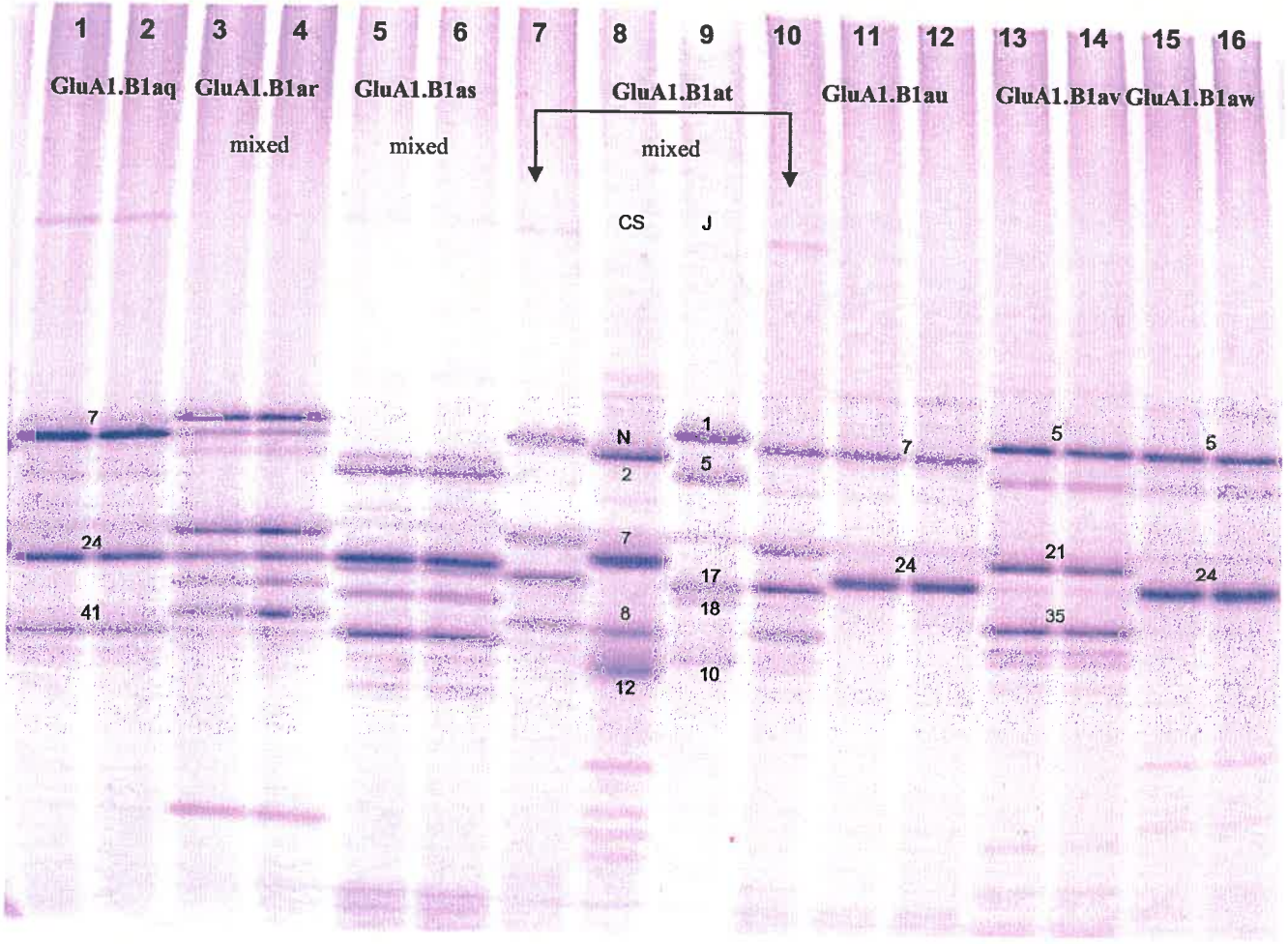

Figure 7. SDS-PAGE (8,5%) of *T.dicoccoides* accessions summarising HMW-GS banding patterns (GluA1.B1 aq to aw). Lanes 1 & 2: AUS 21517; Lanes 3 & 4: AUS 21518; Lanes 5 & 6: AUS 21498; Lanes 7 & 10: AUS 21513; Lane 8: Chinese Spring; Lane 9: Jabiru; Lanes 11 & 12: AUS 26988; Lanes 13 & 14: AUS 23148; and Lanes 15 & 16: AUS 23147.

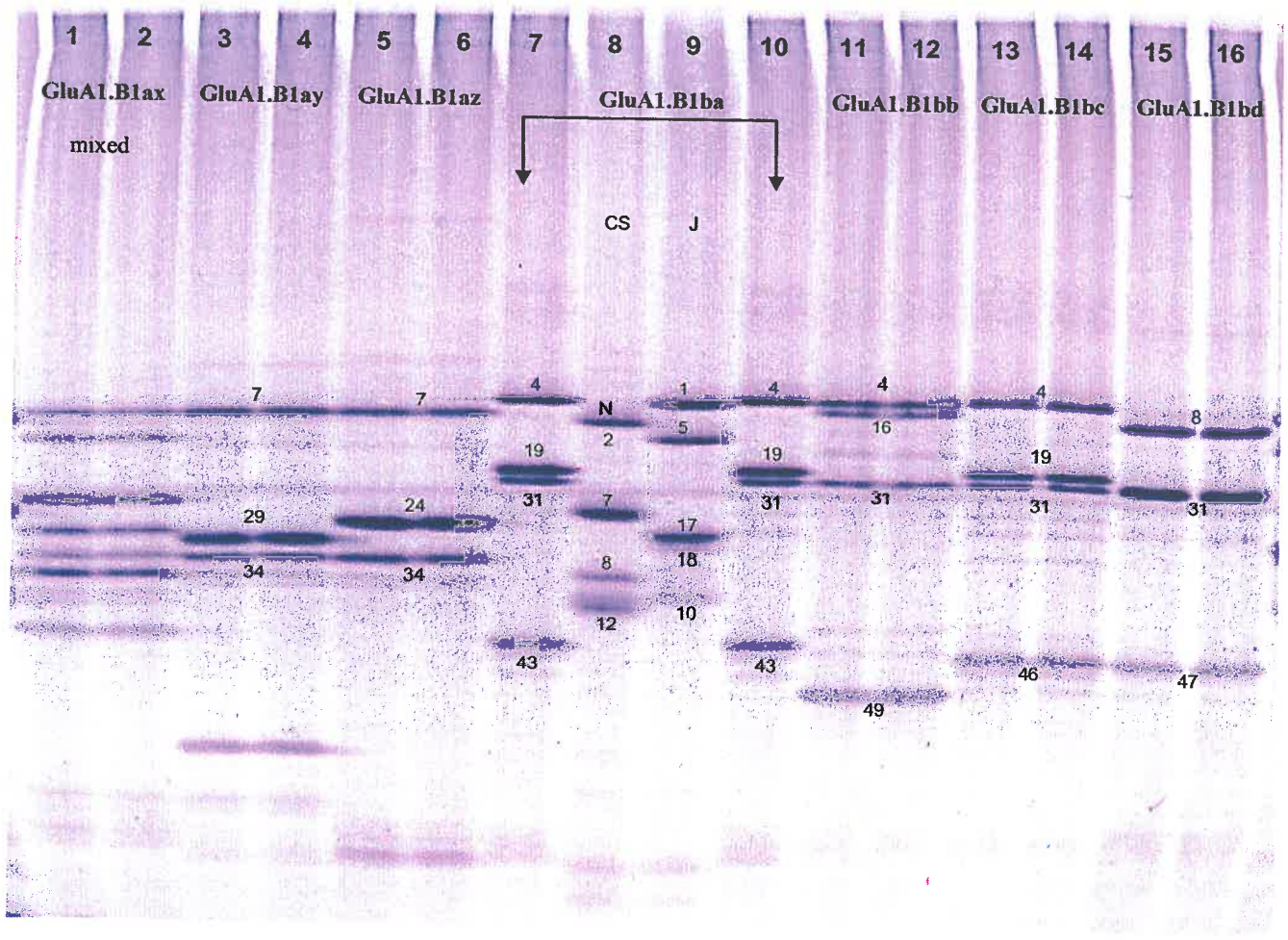

Figure 8. SDS-PAGE (8.5%) of *T.dicoccoides* accessions summarising HMW-GS banding patterns (GluA1.B1 ax to bd). Lanes 1 & 2: AUS 21514; Lanes 3 & 4: AUS 23143; Lanes 5 & 6: AUS 23140; Lanes 7 & 10: AUS 14897; Lane 8: Chinese Spring; Lane 9: Jabiru; Lanes 11 & 12: AUS 17639; Lanes 13 & 14: AUS 23150; and Lanes 15 & 16:AUS 15825.

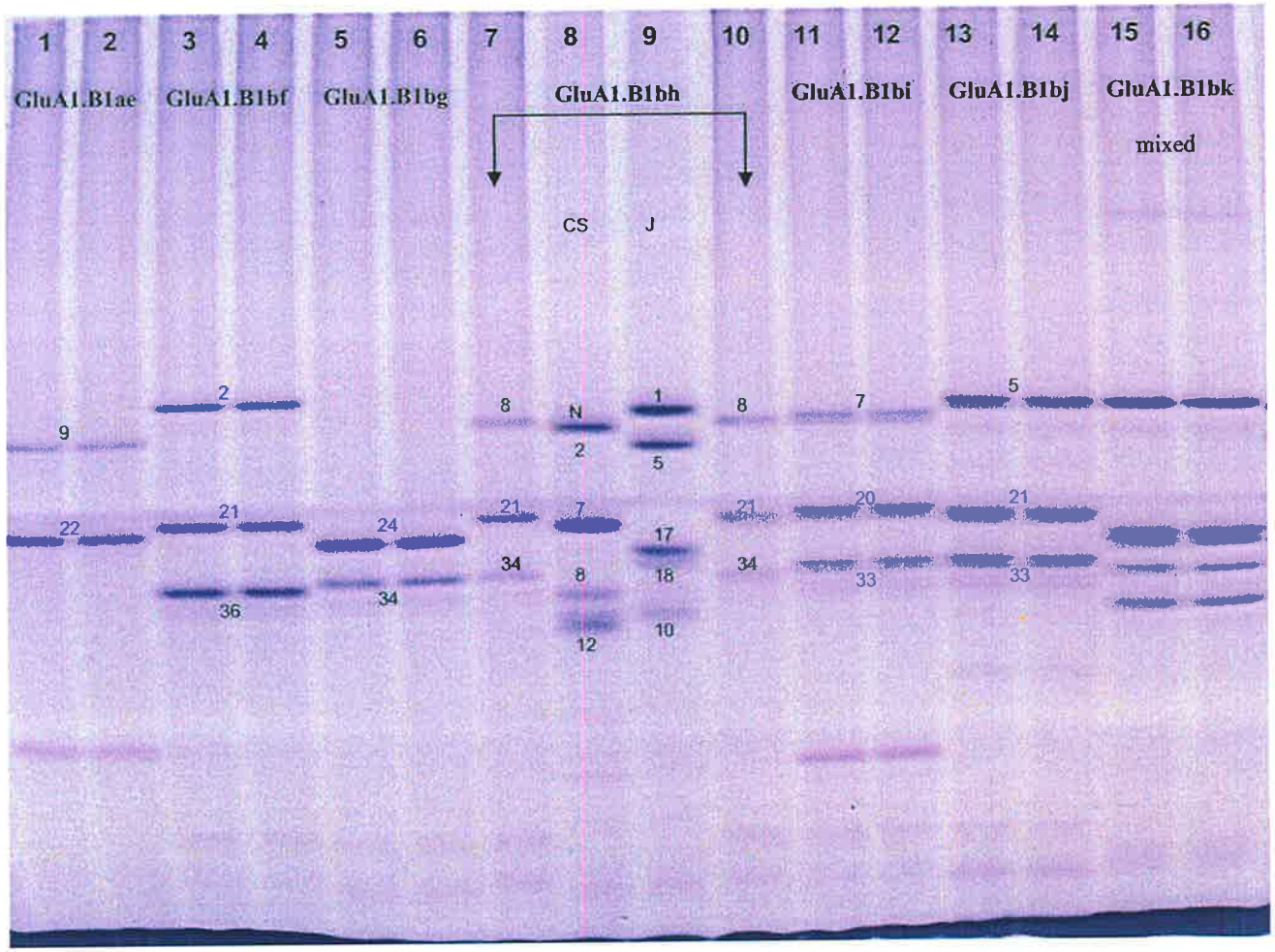

Figure 9. SDS-PAGE (8.5%) of *T.dicoccoides* accessions summarising HMW-GS banding patterns (GluA1.B1 be to bk). Lanes 1 & 2: AUS 217967; Lanes 3 & 4: AUS 27025; Lanes 5 & 6: AUS 27022; Lanes 7 & 10: AUS 27028; Lane 8: Chinese Spring; Lane 9: Jabiru; Lanes 11 & 12: AUS 27029; Lanes 13 & 14: AUS 23160; and Lanes 15 & 16: AUS 2147